Stony Brook University

Mating and Reproductive Patterns

in

Phayre's Leaf Monkeys

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Abstract of the Dissertation

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Patterns of primate mating and reproduction have been studied to (1) better understand species-specific and comparative reproductive life history traits, to (2) evaluate the effect of ecological factors on reproduction, and to (3) investigate patterns of mating in relation to reproductive state and fertility. Information on reproductive parameters such as cycle length, gestation length, and the likelihood of ovulation and conception not only give us a basic understanding of the reproductive system of a species, but allow us to better interpret female behavior in the context of reproductive state (e.g., Wallen, 2001). Many of these reproductive traits are influenced by ecological factors and their effects on food availability (Knott, 2001). It is well-established that fluctuating levels of food availability can affect female reproductive traits via their impact on nutritional state (Bronson, 1985; Ellison, 2003). However, the effect of phytosteroids on reproduction has received increasing attention in recent years due to the finding that consumption of plants in the genus *Vitex* can lead to increased progestin levels and impaired reproductive function in wild primates (Higham et al., 2007;

Thompson et al., 2008). Hence there may be a two-fold effect of food availability on reproduction.

Unlike reproductive parameters, mating patterns are often influenced by factors outside of ecology. These factors might include male quality (Clutton-Brock and McAuliffe, 2009; Stumpf and Boesch, 2005), infanticide risk (Hrdy and Whitten, 1987; van Noordwijk and van Schaik, 2000) and female dependence on male assistance (e.g., infant care, intervention in conflicts; reviewed in Soltis, 2002). While females benefit from honestly advertising fertility to mate with high quality males, they also benefit from concealing fertility from other males such that paternity assurance is low, and males are less likely to commit infanticide (Stumpf and Boesch, 2005; van Schaik et al., 2000). Confusing paternity might also increase the chances that males will offer assistance (e.g., infant care) in exchange for mating (Taub, 1980; van Schaik and Paul, 1996). Given these competing "choices," females may adopt a strategy where they mate outside of the fertile period, but offer some accurate information on the timing of fertility and show preference towards high quality males during such fertile times (van Schaik et al., 2000).

I investigated patterns of mating and reproduction in wild female Phayre's leaf monkeys (*Trachypithecus phayrei crepusculus*) at Phu Khieo Wildlife Sanctuary (Thailand). In this species, reproduction is not strictly seasonal, but the majority of conceptions occur during periods of abundant rainfall associated with greater food availability (Koenig and Borries, unpublished data). Although a high lactation to gestation ratio suggests that the incentive for males to commit infanticide is high (van Schaik, 2000b), infant deaths due to male aggression have never been documented. This study had three major goals: (1) First, I validated the use of fecal hormone metabolites (estrogens and progestins) to assess reproduction and provided the first estimates of reproductive parameters such as gestation and menstrual cycle length (including the separate follicular and luteal phases). (2) I then examined the effect of phytosteroids and nutrition on female reproduction. Because I found evidence of seasonal patterns of fecal progestins, I hypothesized that these patterns were associated with *Vitex* availability. I then investigated whether elevated progestins affected cycle length, receptive period characteristics, or the probability of conception, and whether changes in physical condition explained similar patterns. (3) Finally, I examined patterns of sexual behavior

and mate choice during fertile (around ovulation) and non-fertile receptive periods (during cycling and gestation). I hypothesized that as in other primate species, female Phayre's leaf monkeys should use a mixed strategy of mating, both concealing fertility by mating outside of the periovulatory period, and conveying some general information of the probability of fertility to increase the chances of mating with the high quality male (the adult male) around ovulation.

Over a 20-month period, I collected fecal samples (N = 2046) for the analysis of estrogen (fE) and progestin (fP) metabolites and behavioral data (*ad libitum* and focal animal sampling) for the analysis of sexual behavior in a group with 11 females and two sexually active males. I supplemented these data with monthly estimates of phenology and female physical condition collected by others (Larney and Borries, unpublished data).

In relation to my first goal, I found that patterns of excreted hormone metabolites could indeed be used to assess reproductive function in Phayre's leaf monkeys. Both fE and fP levels showed expected patterns related to cycling and conception. However, patterns of fP could not be used to adequately assess the end of the luteal phase. Furthermore, a seasonal increase in fP levels obscured biological patterns based on this hormone for part of the study. Using fE patterns instead to estimate reproductive parameters, I found that gestation was 205 days (N = 7). Because previous studies have suggested that seasonally elevated progestins might affect ovarian function, I restricted the analysis of menstrual cycle parameters to the time preceding the seasonal fP elevation, and found a cycle length of 28 days (N = 10), with a 15-day follicular phase (N = 10) and a 12-day luteal phase (N = 12). Estimates of gestation and menstrual cycle parameters were generally consistent with previous studies on colobines. However, the 205-day gestation length adds to a growing body of data suggesting that colobines and cercopithecines differ in their allometric scaling of body mass to gestation length (Borries et al., unpublished data).

In relation to the effect of phytosteroids on reproductive function, I found that periods of elevated progestins coincided with the availability of leaves and fruits from the genus *Vitex*, and these periods were not only associated with longer cycle lengths and follicular phases, but also longer inter-mating intervals (intervals preceding receptive

periods) and a greater probability of conception. These results were consistent with previous studies suggesting that elevated progestins and *Vitex* consumption might impair ovarian function and suppress sexual behavior (e.g., Higham et al., 2007), but increase the probability of conception should ovulation occur (Westphal et al., 2004). However, results also show that nutritional status cannot be ruled out as an additional factor explaining reproductive patterns, at least related to conception. Specifically, when ovulations occurred, conceptions were also more likely during months when physical condition was increasing, and these months overlapped the period of elevated progestins. Because food availability in general might affect reproduction via both avenues - nutrition and phytosteroid consumption - these results suggest that the effects of the two may be difficult to disentangle (Knott, 2001). Therefore, future results, particularly on phytosteroids, must be interpreted with caution until more detailed information is available for the exact proximate mechanisms mediating plant consumption, their effect on endogenous steroids, and reproduction.

Finally, I investigated patterns of mating and mate preference in relation to fertile (periovulatory) and non-fertile periods (cycling and post-conceptive), focusing specifically on the phase of the study when two males (one adult and one subadult) were sexually active. I found that consistent with studies on other primates, females were receptive throughout cycling and even during gestation. Receptive periods were around four days long (N = 59), with an average inter-mating interval of 17 days (N = 46). Neither the length of the receptive period nor the inter-mating interval differed when compared across fertile receptive periods, non-fertile cycling receptive periods, and post-conceptive receptive periods. However, receptive periods overlapping the periovulatory phase were distinguished from non-fertile receptive periods by higher rates of proceptive and receptive behaviors. Moreover, females were more proceptive towards the adult male during fertile periods, but towards the subadult male during non-fertile periods. These results were consistent with the hypothesis that females balanced a strategy of paternity confusion, coupled with conveying some accuracy on the timing of ovulation. A high lactation to gestation ratio, coupled with the absence of infanticide suggests that non-fertile receptivity might function successfully to confuse paternity and decrease the occurrence of infanticide in this population. In addition, because males have been

observed to care for infants (Koenig et al., 2009) and intervene in female conflicts (Koenig and Borries, unpublished data), females might exchange mating for male services.

Overall, this study provided the first results on reproduction and mating on Phayre's leaf monkeys, and is only one of three studies (Harris and Monfort, 2006; Heistermann et al., 2001b) to do so in a wild colobine species, using non-invasive monitoring of excreted hormone metabolites to monitor reproductive function. The validation of fecal hormones as markers of reproduction provides an important tool for future studies on mating and reproduction in this population. Furthermore, results on basic reproductive parameters, ecological factors affecting reproduction, and female mating patterns found in the present study provide important comparative data to evaluate population and species differences across primates.

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CHAPTER ONE

MATING AND REPRODUCTION IN PRIMATE FEMALES: A STUDY ON A WILD COLOBINE SPECIES

INTRODUCTION & GOALS

Studies on primate female reproductive biology and behavior have historically focused on several different research objectives. (1) First, many studies have aimed to uncover basic information on reproductive parameters such as interbirth intervals, gestation length, and cycle length in order to increase our knowledge of the reproductive biology of specific species (e.g., *Lemuroidea*: Petter-Rousseaux, 1964; *Papio ursinus*: Gilbert and Gillman, 1951; Gillman and Gilbert, 1946; more recently: *Brachyteles arachnoides*: Strier and Ziegler, 1997; *Semnopithecus entellus*: Heistermann et al., 1995) as well as our understanding of comparative primate life history traits (e.g., Barnett and Abbott, 2003; Borries et al., 2001; Martin, 2007; Martin and MacLarnon, 1985). (2) In addition, studies have focused on examining the ecological and social variables affecting female reproductive function, focusing particularly on the impact of nutrition (Bronson, 1985; Ellison, 2003; Knott, 2001; Koenig et al., 1997), and social stress (Abbott, 1991; Wasser, 1983) on female fertility. (3) And finally, studies on reproduction and sexual behavior have examined patterns of mating from the perspective of sexual selection (Heistermann et al., 2001b; Hrdy, 1979; Stumpf and Boesch, 2005; van Noordwijk and van Schaik, 2000). With reference to this particular objective, much attention has been paid to the observation that primates have "concealed ovulation", or extended receptivity (reviewed in Manson, 1986; Pawlowski, 1999; Small, 1988; van Noordwijk and van Schaik, 2000). Two major aspects of this research have been to examine the proximate mechanisms underlying reproductive behavior (e.g., Glick et al., 1982; reviewed in Wallen, 2001), and the adaptive functions that might explain extended female receptivity (e.g., Hrdy, 1979; Small, 1988; reviewed in van Noordwijk and van Schaik, 2000).

Because humans share a close evolutionary history with non-human primates (Fleagle, 1999), all three of these objectives – obtaining comparative information on reproductive parameters, understanding the variables that affect reproduction, and examining female sexual strategies - enhance our understanding of the reproductive physiology and behavior in humans (Gangestad and Thornhill, 2008; Pawlowski, 1999). Furthermore, knowledge of basic reproductive parameters and the socioecology of female reproduction is integral to designing captive management and conservation strategies for endangered primate species (see Brown, 2000; Cockrem, 2005).

While early studies on primate reproduction and mating behavior were conducted in captivity (e.g., Rowell and Chalmers, 1970), increasing studies on free-ranging (e.g., Loy, 1971; Möhle et al., 2005; Small, 1990; Takahata, 1980) and/or wild populations (e.g., Altmann et al., 1977; Borries et al., 2001; Deschner et al., 2004; Smuts, 1985; Sommer et al., 1992; Strier and Ziegler, 1997), particularly in the last 30 years, have expanded our breadth of comparative knowledge, and enhanced our understanding of how reproduction is shaped by ecology and sexual selection in a naturalistic setting.

Until recently, however, the precision of reproductive estimates and the feasibility of assessing particular hypotheses have been limited. This limitation has been primarily due to the inability to assess reproductive *physiology* without the use of invasive methods such as darting or trapping (e.g., Sapolsky and Share, 1998). Although rough estimates of cycling parameters, conception, and gestation length can be made using markers such as perineal swellings (e.g., Altmann et al., 1977), menstruation (e.g., Zuckerman, 1937), and behavior (e.g., Sommer et al., 1992), the majority of primates do not have perineal swellings (Sillen-Tullberg and Møller, 1993), and menstruation is difficult to observe in naturalistic conditions. Furthermore since primates, and particularly, haplorrhines, are known for their capacity for receptivity outside of the periovulatory period (Wallen, 2001), neither estimates based on perineal swellings or behavior are precise (Brauch et al., 2007; Higham et al., 2008a), and more recent studies have shown that either or both may be used by females as a sexual strategy to mask the true timing of ovulation (Barelli et al., 2007; 2008; Deschner et al., 2004; Heistermann et al., 2001b). Indeed, the degree to which primates use either sexual behavior or swellings as honest or dishonest signals of fertility can only been assessed with the help of hormonal estimates of ovulation.

Finally, without knowledge of reproductive physiology, researchers are unable to document the proximate mechanisms linking ecology, reproduction, and sexual behavior in the wild populations.

In this regard, the advent of non-invasive methods to monitor reproductive steroid levels has played a key role in expanding several avenues of research in the wild (Whitten et al., 1998; Ziegler and Wittwer, 2005). Specifically, the ability to measure steroid metabolites in excreted matter has (1) increased our ability and our precision to measure reproductive parameters, (2) given researchers the tools to evaluate whether females use either behavior or perineal swellings as signals related to sexual strategies (Barelli et al., 2008; Deschner et al., 2004), and (3) provided the means to investigate underlying proximate mechanisms controlling sexual behavior and reproduction (Brauch et al., 2007; Higham et al., 2009).

With regards to the latter, recent studies have uncovered novel ecological and dietary factors (Higham et al., 2007; Thompson et al., 2008), in addition to general food availability and its impact on nutrition (Bronson, 1985; Ellison, 2003), that might affect female reproductive physiology. Specifically, the observation that dietary phytosteroids can affect female fertility via its interference with endogenous steroids has revealed an addition layer of variables that might affect the timing of reproduction in wild populations (Higham et al., 2007; Thompson et al., 2008). In sum, the use of non-invasive steroid monitoring in wild populations has contributed substantially to our understanding of the ecological and evolutionary factors influencing female reproduction and mating patterns in primates.

As part of this tradition, I conducted a study on female reproduction and sexual behavior in a group of wild Phayre's leaf monkeys (*Trachypithecus phayrei crepusculus*) in Phu Khieo Wildlife Sanctuary, Thailand. Over the course of 20 months, I collected fecal samples to monitor female reproductive hormones, and data on sexual behavior to study mating patterns and mate choice. The primary objectives of this study were: (1) to validate the use of fecal steroid monitoring in *T. phayrei crepusculus* as a tool to assess reproductive parameters such as gestation length, cycle length, and the different components of the menstrual cycle (ie. luteal and follicular phases) (Chapter 2), (2) to investigate the potential role of nutritional and dietary factors influencing female

reproduction (Chapter 3), and (3) to examine the proximate and ultimate factors shaping receptivity outside of the fertile period by evaluating temporal patterns of receptivity, the "honesty" of female sexual behavior, and female mate preferences during fertile versus non-fertile receptive periods (Chapter 4).

COLOBINE REPRODUCTION AND MATING PATTERNS

Phayre's leaf monkeys belong to the family Colobinae, a taxon composed of 10 genera (*Colobus, Nasalis, Piliocolobus, Presbytis, Procolobus, Pygathrix, Rhinopithecus, Semnopithecus, Simias, Trachypithecus*) (Osterholz et al., 2008; Ting et al., 2008). Colobines are best known for their specialized dietary adaptations to eating leaves (Chivers and Hladik, 1980). The majority of species inhabit tropical environments in Africa and Asia (Struhsaker and Leland, 1987), with the exceptions being Hanuman langurs, which can be found in urban and desert environments in India (Koenig and Borries, 2001), and Sichuan golden monkeys, which are found in temperate regions in China (Chen et al., 1983).

Although closely related to cercopithecines, colobines are generally characterized by comparatively smaller social groups, with mating systems in most species characterized by polygynous one-male, multi-female groups and/or promiscuous multi-male, multi-female units with no more than four to five adult males (Struhsaker and Leland, 1987; but see Koenig and Borries, 2001; Korstjens and Noe, 2004; Struhsaker, 1975). This is in contrast to many well-known cercopithecine species, where large, promiscuous multi-male/multi-female groups are the norm (Melnick and Pearl, 1987). Yet another feature that distinguishes colobines from cercopithecines is the general absence of perineal swellings (Sillen-Tullberg and Møller, 1993; Struhsaker and Leland, 1987). Indeed, conspicuous perineal swellings have thus far only been found in a couple of species (*Colobus badius, Procolobus verus*: Struhsaker, 1975), most of which live in larger multi-male groups, and are thus the exception, rather than the rule among colobines (Struhsaker and Leland, 1987).

Reproductive estimates based on patterns of menstruation and physiology have yielded a mean cycle length of 22-30 days (Gibson and Chu, 1992; Harris and Monfort, 2006; He et al., 2001; Ruempler, 1998; Ziegler et al., 2000) and a gestation length of 180-

211 days (Borries et al., 2001; Dasilva, 1989; Harris and Monfort, 2006; Rudran, 1973b; Shelmidine et al., 2009). As in many other primate species (reviewed in Brockman and van Schaik, 2005) annual birth peaks have been documented in several colobine species (reviewed in Struhsaker and Leland, 1987; see references that follow), and in many of these, birth seasonality has often been explained by a greater probability of conception during months when increased food availability improves female body condition (*Rhinopithecus bieti*: Xiang and Sayers, 2009; *Semnopithecus entellus*: Koenig et al., 1997; *Trachypithecus leucocephalus*: Jin et al., 2009; *T. phayrei*: Borries and Koenig, 2005). The few studies focusing on sexual behavior have shown that colobines exhibit the extended female receptivity typical of haplorrhine primates (Borries et al., 2001; Harris and Monfort, 2006; Korstjens and Noe, 2004; Sommer et al., 1992), with sexual behavior often occurring outside of the periovulatory period (see above references) and even during gestation (Borries et al., 2001; Gorzitze, 1996; Newton, 1987; Shelmidine et al., 2009). In fact, the observation of post-conceptive mating coupled with the prevalence of infanticide in wild Hanuman langurs led Hrdy (Hrdy, 1974; 1979) to hypothesize that extended receptivity in primates has evolved to confuse paternity as an adaptive strategy to decrease the risk of infanticide. This strategy might be conditional, occurring only when a new male takes over a group (e.g., Pazol, 2003), or constant, as might be the case in a multi-male group, where all males other than the sire may benefit from committing infanticide if paternity is concentrated and known (reviewed in Hrdy and Whitten, 1987; van Noordwijk and van Schaik, 2000). Indeed, because infanticide has been documented in several colobine species (*Colobus guereza*: Harris and Monfort, 2003; Oates, 1977; Onderdonk, 2000; *Colobus vellerosus*: Teichroeb and Sicotte, 2008; *Presbytis senex*: Rudran, 1973b; *Presbytis thomasi*: Steenbeck, 2000; *Rhinpithecus bieti*: Xiang and Grueter, 2007; *Semnopithecus entellus*: Borries, 1997; Hrdy, 1974; Mohnot, 1971; Sugiyama, 1965), it seems plausible that it has played an important role in shaping female reproductive strategies in this taxon.

More recently, a study on wild Hanuman langurs incorporating endocrine measures for estimates of ovulation and fertility has shown that although females do indeed exhibit higher rates of receptivity around ovulation, receptive periods were variable in length, often extending beyond the fertile phase, and rates of proceptivity

were not significantly different across the cycle (Heistermann et al., 2001b). These results support the hypothesis that sexual behavior and extended receptivity might function to confuse paternity so that that males will be less likely to commit infanticide.

To date, however, only two studies (Harris and Monfort, 2006; Heistermann et al., 2001b), including the one discussed above, have used endocrine measures to examine colobine reproduction and sexual behavior in the wild. Hence, the present study on Phayre's leaf monkeys will contribute substantially to current knowledge, especially in addressing proximate mechanisms associated with reproduction and behavior, and female mating strategies across fertile and non-fertile periods.

Phayre's Leaf Monkeys

Phayre's leaf monkeys (*Trachypithecus phayrei crepusculus*) belong to the Asian clade of colobines and are found specifically in Southeast Asia (Ting et al., 2008). Although there have been two field studies carried out on this species (Phu Khieo Wildlife Sanctuary, Thailand: Borries et al., 2008; Koenig et al., 2004; Tripura, India: Gupta and Kumar, 1994) the majority of our knowledge comes from Phu Khieo Wildlife Sanctuary (PKWS), in Thailand. This study was established and is currently run by Andreas Koenig and Carola Borries. Major research topics that have been investigated at PKWS include: feeding ecology (Koenig et al., 2008), female social relationships (Koenig et al., 2004), male-infant relationships (Koenig et al., 2009), dispersal patterns (Borries et al., 2004; Koenig et al., 2006) and factors shaping female fitness (Borries et al., 2008; Larney and Koenig, 2007). In addition to behavioral and demographic monitoring, research at PKWS has incorporated measures of food availability, physical condition, and genetics as standard protocol (Borries, Koenig, Larney, Ossi, unpublished data). The present study on female reproduction and mating behavior adds endocrine monitoring to this list of measures. As such, it complements previous methods which together, provide a more well-rounded study of the proximate and ultimate mechanisms influencing behavior in this primate population.

To date, results from PKWS have shown that the social organization of *T. phayrei crepusculus* varies from one-male to multi-male, with multi-male groups containing up to five adult males (Borries et al., 2008). Interbirth intervals are known to be approximately

two years in length (Borries et al., 2008), and annual conception peaks related to food availability, based on demographic estimates of gestation length, have been shown (Borries et al., 2005). Currently however, more precise physiological estimates of conception, gestation lengths, and cycling patterns in particular, are unavailable.

Despite nearly a decade of research, infanticide has never been documented (Borries and Koenig, unpublished), making this population somewhat exceptional among colobines. Furthermore, unlike most other colobines (but see Xiang et al., 2009), males often affiliate with infants, sometimes even carrying them (Koenig et al., 2009). This observation is interesting, given previous hypotheses that extended receptivity might also be expected if females mate with males in return for some degree of infant care (Taub, 1980). Males are generally patrilocal (Koenig et al., 2006), and young adult males have been observed to reach alpha rank early in maturity (Borries and Koenig, unpublished data). Hence, females may view males both as potential mates and infanticidal threats early in a male's career, even prior to reaching full adulthood.

Seasonality in conceptions, coupled with these unusual socioecological characteristics, make Phayre's leaf monkeys at Phu Khieo Wildlife Sanctuary an interesting species and population in which to carry out a study on female reproduction and mating strategies.

CHAPTER OUTLINE

In the following section, I outline the major objectives of each thesis chapter, providing a brief background on previous studies and hypotheses where relevant. More extensive details will be provided in the chapters themselves. Note that while I am the primary author, all thesis chapters were collaborative efforts with coauthors who either contributed data or provided valuable ideas in the design of the study and/or the interpretation of the results. Henceforth, I will use the pronoun "we" when discussing all subsequent chapters.

Chapter Two

In chapter two, we use fecal steroid monitoring to determine reproductive parameters in female Phayre's leaf monkeys. Specifically, our objectives are: (1) to

validate the use of fecal estrogen and progesterone metabolites in order to assess reproductive function in Phayre's leaf monkeys, (2) to present first data on reproductive parameters, including gestation length, cycle length, and lengths of the different component phases (ie. follicular vs. luteal) of the menstrual cycle, and (3) to compare these parameters to previous results for primates, paying particular attention to cercopithecines and colobines.

Chapter Three

In chapter three, we examine the potential role of dietary factors and nutrition in influencing female reproductive function. Two recent studies in wild primate populations have found that dietary phytosteroids, particularly phytoprogestins, may influence female reproductive physiology, perhaps also influencing fertility and the timing of female reproduction (Higham et al., 2007; Thompson et al., 2008). Results from both studies found that females exhibited elevated progestin levels associated with the consumption of plants from the genus, *Vitex*. Coupled with captive studies showing that phyosteroids can interfere with ovulation in primate females (e.g., Trisomboon et al., 2006b; Trisomboon et al., 2004) and previous suggestions that phytosteroids might influence conception in wild primate populations (Garey, 1991; Wallis, 1997; Whitten, 1983), we investigate whether phytosteroids might also be a factor influencing reproductive function in Phayre's leaf monkeys. However, because food consumption might also influence reproduction via its effects on nutritional status (Bronson, 1985; Ellison, 2003), we also evaluate whether reproductive patterns could be explained by patterns of female physical condition.

We specifically have four main objectives: (1) First, we examine whether fecal progestin levels in female Phayre's leaf monkeys exhibit a seasonal pattern. (2) Second, we investigate whether these patterns were consistent with a plant-based hypothesis, particularly, with the consumption of *Vitex*. Because these results were not expected, we did not have feeding data on specific food items. Therefore, we investigated this hypothesis indirectly by testing whether fecal progestin patterns were linked to the availability of *Vitex* species. (3) Third, we evaluate whether periods of elevated progestins influence ovulation, reproductive behavior, and/or conception. (4) And finally,

we determine whether the same reproductive patterns are consistent with changes in female physical condition.

Chapter Four

In chapter four, we examine patterns of female sexual behavior, exploring the underlying hormonal mechanisms that influence behavior and the timing of mating and mate choice in relation to fertility. In contrast to other mammals, primate, and particularly haplorrhine, sexual behavior is thought to be less tightly coupled to the periovulatory period and to the hormonal changes that occur at this time (Wallen, 2001). However, although females of many primate species exhibit receptivity outside of the fertile period (van Schaik et al., 2000), receptivity is generally more common in constrained periods of time, often related to an increase in the level of estrogens relative to progestins (e.g., perimenstrual receptivity: Loy, 1970; post-conceptive receptivity: Engelhardt et al., 2007). Furthermore, a vast number of studies also show that rates of proceptive and receptive behaviors peak around ovulation (reviewed in Dixson, 1998). From a proximate perspective, these patterns suggest that sexual behavior is not completely independent of ovarian hormones (Wallen, 2001). From an ultimate perspective, a peak in receptivity around ovulation, coupled with the capacity for continuous receptivity, is thought to reflect a mixed strategy that females might employ to increase individual fitness (Stumpf and Boesch, 2005). On the one hand, sexual behavior that is honest would function to increase the chances that females mate with higher quality males during times of peak fertility (Clutton-Brock and McAuliffe, 2009). On the other hand, some degree of mating outside of the fertile period might help females confuse paternity so that other males might be less likely to commit infanticide (Hrdy, 1979), and more likely to provide services for the female such as infant care (Taub, 1980) or support in conflicts (Smuts, 1985; reviewed in Soltis, 2002; van Noordwijk and van Schaik, 2000). Therefore, one would predict that females might be more proceptive and receptive toward dominant males during fertile periods (Stumpf and Boesch, 2005) but show more preference towards subordinate males during non-fertile periods. Furthermore, in order to facilitate paternity confusion, non-fertile receptivity might be unpredictable.

Although infanticide has never been documented in Phayre's leaf monkeys (Koenig and Borries, unpublished data), females are characterized by a high lactation to gestation ratio, suggesting that the incentive for males to kill unrelated infants could be high (van Schaik, 2000b). Furthermore, adult males are known to intervene in female conflicts and affiliate with infants, providing some degree of "paternal" care (Koenig et al., 2009). Hence, females have several potential incentives for paternity confusion.

Therefore, in chapter four, we use behavioral data and hormonal estimates of ovulation to investigate patterns of receptivity in our study group, during a time period when one adult and one subadult male were mating. We compare sexual behavior during fertile periods (around ovulation) to non-fertile periods (cycling and during gestation). We have three primary objectives: (1) First, we examine temporal patterns of receptivity during cycling and gestation and determine if these patterns are consistent with a hormonally-based explanation. (2) Second, we determine if female receptivity is "honest," by comparing characteristics (i.e. receptive period length, inter-mating interval, and rates of proceptive and receptive behaviors) of fertile and non-fertile receptive periods. We specifically predict that receptive periods during fertile and non-fertile periods should exhibit no difference, if female strategies are heavily biased towards paternity confusion. (3) Finally, we examine female preference for different males during fertile vs. non-fertile receptive periods, predicting that female proceptivity and receptivity will be more often directed at the adult male during fertile periods and the subadult male during non-fertile periods.

Chapter Five

In chapter five, I provide a summary and synthesis of major results and suggest directions for future research. I focus on several methodological and theoretical issues that were either treated with less detail or were unaddressed in previous chapters. These issues include: (1) optimizing assay protocols, (2) appropriate species means for comparative analyses, (3) future phytosteroid research, (4) functional interpretations of non-fertile matings, and (5) potential variation in male knowledge of female fertility.

CHAPTER TWO

REPRODUCTIVE CHARACTERISTICS IN WILD PHAYRE'S LEAF MONKEYS

ABSTRACT

An understanding of basic female reproductive characteristics such as cycle and gestation length are crucial to assessing fertility, interpreting female behavior, and designing appropriate conservation and captive management plans for endangered species. In primates such as colobines, estimates of reproductive parameters, particularly related to cycling, are dependent on the analysis of reproductive hormones, as morphological signs of receptivity are lacking. Here we use fecal hormone analysis to characterize cycle patterns and gestation length in a group of wild Phayre's leaf monkeys (*Trachypithecus phayrei crepusculus*) in Phu Khieo Wildlife Sanctuary, Thailand. We found that both fecal estrogen (fE) and progestin (fP) levels showed clear biological patterns indicative of ovulation and conception. However, due to a strong seasonal effect on fP that obscured biological patterns during part of the year, fE patterns were more useful in assessing cycle and gestation characteristics. We found a mean cycle length of 28 days (N = 10), with follicular and luteal phases of 15 (N = 10) and 12 days (N = 12) respectively. On average, females cycled 3.57 ± 0.43 (N = 7) times until conception. Average gestation length was 205.3 ± 1.41 days (N = 7), with fE levels increasing over the course of pregnancy. Overall, the reproductive characteristics found for Phayre's leaf monkeys were consistent with previous studies on colobines, suggesting that fecal hormone monitoring, particularly for fE metabolites, can provide useful information on reproduction in this species.

INTRODUCTION

Measuring reproductive steroid levels in mammalian females provides basic information on female reproductive characteristics such as cycle length and gestation (e.g., *Ceratotherium*: Patton et al., 1999; *Chinchilla*: Busso et al., 2007; *Colobus*: Harris and Monfort, 2006; *Equus*: Asa et al., 2001; *Mustela*: Young et al., 2001; *Papio*: Beehner et al., 2006; *Sarcophilus*: Hesterman et al., 2008). Knowledge of such characteristics (i.e., cycling patterns, gestation length, and reproductive state) is crucial to assessing fertility, understanding female behavior (del Castillo et al., 2005; Maestripieri, 1999; Ramirez et al., 2004) and sexual strategies in particular (e.g., Deschner et al., 2004; Engelhardt et al., 2007), and examining differences in life history strategies within (e.g., Borries et al., 2001) and across species (Barnett and Abbott, 2003; Harvey et al., 1987; Martin and MacLarnon, 1985). Furthermore, a fundamental understanding of female reproductive physiology is an imperative first step in developing management and conservation plans for endangered populations (e.g., Brown, 2000; Cockrem, 2005).

In primates, the monitoring of estrogen and progesterone metabolites in wild populations has successfully been applied to cercopithecines (*Macaca fascicularis*: Engelhardt et al., 2005; *M. fuscata*: Fujita et al. 2004; *M. sylvanus*: Brauch et al., 2007; Möhle et al., 2005; *M. tonkeana*: Aujard et al., 1998; *Papio cynocephalus*: Beehner et al., 2006, Gesquiere et al., 2007; Higham et al. 2008a) and hominoids (*Hylobates lar*: Barelli et al., 2007; *Pan troglodytes*: Deschner et al., 2004; Thompson, 2005; *Pongo pygmaeus*: Knott, 2001). Both groups have also been studied extensively in captivity (e.g., cercopithecines: *Macaca assamensis*: Wehrenberg et al. 1980; *M. fascicularis*: Shideler et al., 1993; *M. fuscata*: Nigi and Torii 1983; *M. mulatta*: Weick et al., 1973; hominoids: *Gorilla gorilla*: Czekala et al., 1988; Nadler et al., 1983; *Hylobates lar*: Nadler et al., 1993; *Pan troglodytes*: Nadler et al., 1987; *Pongo pygmaeus*: Nadler et al., 1984), yielding a comprehensive understanding of the reproductive biology of these species. With the exception of captive studies on the callitrichines (e.g., French et al., 1996; Heistermann and Hodges, 1995; Ziegler et al., 1996), however, fewer studies have been conducted on other primate groups, particularly in the wild. For instance, within haplorrhines, we know comparatively little about New World Monkeys (*Alouatta pigra*: van Belle et al. 2009; *Brachyteles arachnoides*: Ziegler et al., 1997; *Cebus capucinus*:

Carnegie et al., 2004), or within catarrhines, about colobines (*Colobus guereza*: Harris and Monfort, 2006; *Semnopithecus entellus*: Ziegler et al., 2000). Similarly, estimates of reproductive parameters based on hormonal estimates have only been assessed for a handful of strepsirrhines in the wild (*Eulemur fulvus*: Ostner and Heistermann, 2003; *E. mongooz*: Curtis et al., 2000). Because many of these under-represented taxa also show no conspicuous visual signs of estrus (Nunn, 1999; Sillen-Tullberg and Møller, 1993), estimates of cycle length based on sexual swellings (e.g., Altmann et al., 1977) are not possible. Furthermore, because primate sexual behavior commonly occurs outside of the periovulatory phase (Wallen, 2001), estimates of cycling based solely on behavior may be less precise than hormonally-based assessments.

In this study, we analyze fecal estrogen (fE) and progesterone (fP) metabolites to examine female reproductive characteristics in a wild group of Phayre's leaf monkeys (*Trachypithecus phayrei crepusculus*) in Phu Khieo Wildlife Sanctuary, Thailand. Phayre's leaf monkeys belong to the family Colobinae, a group known for their folivorous dietary specialization (Chivers and Hladik, 1980). Although the phylogenetic systematics of colobines is still debated, the general consensus is that the family is composed of 10 genera: three belonging to an African clade (*Colobus, Piliocolobus Procolobus*) and seven belonging to an Asian clade (*Nasalis, Presbytis, Pygathrix, Rhinopithecus, Semnopithecus, Simias, Trachypithecus*) (reviewed in Osterholz et al., 2008; Ting et al., 2008). The genus *Trachypithecus* is estimated to contain between 10 and 17 different species (Brandon-Jones et al., 2004; Groves, 2001), several of which are listed as critically endangered (e.g., *T. delacouri, T. francoisi*) or endangered (e.g., *T. germaini, T. phayrei;* IUCN Red List: http://www.iucnredlist.org/). Despite this fact, there currently exists only one study on the reproductive physiology of the entire genus (*T. francoisi*: Wang et al., 2006), and this study did not report any results pertaining to basic reproductive parameters. Furthermore, within colobines in general, endocrine data on the menstrual cycle (e.g., menstruation, follicular, ovulatory, and luteal phases) are available for only five of the 10 genera (*Colobus*: Harris and Monfort, 2006; *Pygathrix*: Heistermann et al., 2004; *Rhinopithecus*: Hama et al., 1997; He et al., 2001: Yan and Jiang, 2006; *Semnopithecus*: Ziegler et al., 2000; *Trachypithecus*: Wang et al., 2006).

Therefore, additional studies on the reproductive physiology of colobines as a whole, and of *Trachypithecus* in particular, are long overdue.

The majority of information known about Phayre's leaf monkeys derives from a single population studied in Phu Khieo Wildlife Sanctuary, Thailand. Research at this site indicates that individuals live in groups of three to 33 individuals (Borries et al., 2008; Koenig et al., 2004). The majority (58%) of groups are multi-male, containing two to five adult males, with the remaining groups (42%) uni-male. Mating can occur year round, but a birth peak between November and April (Borries et al., 2007, 2008, in prep) suggests a corresponding mating peak between April and October (see methods). Although sexual swellings have been documented for a related species in captivity (*Trachypithecus cristatus*: Shelmidine et al., 2007), they are relatively inconspicuous compared to the swellings of macaques and baboons (reviewed in Nunn 1999), and do not indicate fertility, but rather relate to gestation. While we have occasionally observed swellings in Phayre's leaf monkeys, they are similarly inconspicuous and rare, even in the mating season. Therefore, endocrine monitoring in this species is essential to provide information on menstrual cycle characteristics.

Our specific research goals are the following: (1) to describe the pattern of fE and fP levels during the mating season in Phayre's leaf monkeys, (2) to determine cycle length, the different phases of the cycle (i.e., follicular, luteal), and gestation length, and (3) to compare these reproductive characteristics to what is known for other primates, particularly cercopithecines and colobines.

METHODS

Study Site

Research was carried out at Phu Khieo Wildlife Sanctuary, located in northeast Thailand (16°5‘- 35‘N, 101°20‘-55‘E, Chaiyaphum Province, elevation: 300-1300 m above sea level). The sanctuary consists of an area of 1,573 km^2 and is home to a diverse array of vertebrate fauna, including eight species of felids, 10 viverrid species, and two species of raptors (Grassman et al., 2005). In addition to Phayre's leaf monkeys, the sanctuary is home to six other non-human primate species (*Hylobates lar, Macaca arctoides, M. assamensis, M. leonina, M. mulatta, Nycticebus bengalensis*) (Borries et al.,

2002). The study site at Huai Mai Sot Yai is located at 16°27'N, 101°38'E at an elevation of 600-800 m and consists of dry evergreen forest with patches of dry dipterocarp (Borries et al., 2002). Rainfall at the study site averages 1,400 mm per year (Grassman et al., 2005; Kumsuk et al., 1999).

Subjects & Study Period

The research was conducted within an ongoing project run by Andreas Koenig and Carola Borries which began in October 2000. The majority of the data presented here was collected by A.L. (with the help of two trained rangers) and was supplemented by data collected by other project members as part of the standard data collection routine. This study was carried out between February 1, 2005 and September 28, 2006 (20 months), focusing on one group called PB. This group has been fully habituated since the spring of 2004, and demographic data are available from June 2003 onward (Borries & Koenig, unpublished data). At the beginning of the study, PB consisted of one adult male, 11 adult females, and 12 immatures. Over the course of the study, the composition of the group changed due to infant births (N = 7), disappearances (N = 3), and emigration of one adult female (Borries & Koenig, unpublished data). In addition, two juvenile males reached subadulthood in 2006, marked by full descent of the testes. Upon completion of the study, the group thus contained one adult male, two subadult males, 10 adult females, and 14 immatures. All adult females were multiparous at the beginning of the study. Although absolute ages were unknown, relative age estimates based on tooth wear, skin wrinkling, color of eye lens, and nipple length (Hrdy, 1977) suggested that most females (N = 9) were either young or prime adults, with only two females who were older than prime age (Table 2.1).

Our study was conducted in two phases: (1) During Phase I (02/01/05 – 12/12/05) all adult females (N = 11) were followed 12-20 days per month (147 (53.5%) of 275 days, 1662.9 contact hours, 11.3 hours/day), with monthly collection of fecal samples for broad estimates of reproductive state. (2) During Phase II (12/13/05 – 9/28/06), group follows were more frequent to collect fecal samples for estimates of ovulation and conception (265 (90.7%) of days; 3058.9 contact hours, mean = 11.5 hours/day) and

specifically targeted cycling females (N = 7). Data for the present study derived largely from this second phase.

Our goal was to collect a sufficient number of fecal samples from each cycling female from the onset of cycling to parturition. In order to appropriately time the beginning of the study, we estimated the onset of mating in 2006 based on the average interbirth interval and the seasonal pattern of previous birth peaks. Assuming a two-year interbirth interval (IBI) after surviving infants (Borries et al., 2007), we initially expected five adult females to resume cycling (Table 2.2). Two additional females were added to the sample because they began to mate: (1) B3, who was originally assumed to have conceived in early January 2006, and (2) B7, who was not expected to resume cycling during the study period because her infant was too young.

Data from previous years indicates that an annual birth peak occurs between November and April (Borries et al., in prep). Coupled with an estimated 200-day gestation (e.g., Rudran, 1973b; Ziegler et al., 2000) these patterns suggest that a corresponding mating peak was likely between April and October. Assuming three cycles to conception (as reported for many species: Dixson, 1998), we therefore conservatively estimated that mating could begin as early as February. However, the first female was observed to mate on December 13, 2005, and from this point forward we followed the group nearly every day until the last female conceived.

Fecal Sample Collection

Fecal samples were collected *ad libitum*, on a monthly basis from all 11 adult females throughout the course of the study. During Phase II, however, our sample regime became more intensive for the seven females predicted to be cycling. During the first two weeks of this phase (12/13/06 – 12/31/06), sampling focused on two females predicted to cycle earliest (based on previous parturition dates). Sampling was much more sporadic for other cycling individuals during this time (Table 2.2). For all complete months of data collection, fecal samples were collected for 22.3 days/cycling female/month (16 – 35 samples/female/month). In the present paper, we analyzed 1,464 samples collected during cycling, conception, and the initial months of pregnancy from the seven females targeted during Phase II (209.1 samples/female). These data are supplemented by 149 samples

representing four full pregnancies (37.3 samples/female) spanning both Phase I and Phase II.

Entire fecal samples were collected in 30 ml plastic vials (Sarstedt: vial #75.1337.500, lid #76.1340.560), kept on ice in the field, and frozen (-20°C) upon return to the field station (within 2-13 hrs). These samples remained frozen until they were shipped on ice to the Conservation and Research of Endangered Species (CRES) at the Zoological Society of San Diego (Dec. 2006).

Fecal Hormone Analysis

At CRES, samples were lyophilized and sifted through mesh wire (16 x 16 mesh; McMaster-Car: # 9223T82) to remove vegetative matter. Samples were then transported to the Core Assay Facility in the Department of Psychology at the University of Michigan for further analyses. At the University of Michigan, samples were doubly extracted following protocols established by Wasser et al. (1994). In brief, for each extraction, a 0.1 g portion of sample was added to 95% ethanol in 8 ml plastic test tubes (Sarstedt: #60.542.007). The tubes were then vortexed for one minute, boiled in a water bath (90°C) for 20 minutes, and centrifuged at 1,500 rpm for an additional 20 minutes. After centrifugation, the supernatant was decanted into 16x100 mm test tubes (Fisher: #14-961-29). This process was repeated twice, at the end of which the fecal matter was discarded and the supernatant dried down under nitrogen gas. Extracted samples were reconstituted in 100% ethanol, transferred into 2ml microcentrifuge tubes (Sarstedt: #72.694.007) and stored in the freezer (-20°C) until analysis. Extraction of radiolabeled progesterone and estrogen yielded recoveries of 86.7 ± 3.8% (N = 10) and 91.5 ± 0.8% (N = 10), respectively.

Progesterone and estrogen metabolites were analyzed using radioimmunoassay (RIA). Progesterone metabolites were assessed with a double antibody RIA protocol (Guesquiere et al. 2008: http://www.princeton.edu/~baboon/publications/Altmann_lab_protocols_Jan08.pdf). This RIA uses a progesterone primary antibody (CL425: C. Munro, UC Davis) diluted 1:12,000, coupled with commercial progesterone tracer and standards (Pantex: #137 TRA, #137 CAL, #137 CALO), and a secondary antibody (Goat Anti-mouse IgG, Equitech Bio: #GAMG-0100). The CL 425 antibody is a

monoclonal antibody raised against 4-Pregnen-3α-ol-20-dione (P4) hemisuccinate:bovine serum albumin (Grieger et al., 1990). It is known to cross-react with a variety of progesterone metabolites (e.g., 4-Pregnen-3α-ol-20-one: 188%; 5α-Pregnan-3ß-ol-20-one: 94%; pregnanediol: <0.1%; see Graham et al. 2001) and has been successfully applied to study fecal progestins in a variety of mammals (e.g., Graham et al., 2001; North and Harder, 2008). Fecal estrogen metabolites were assessed using a commercial estradiol (E2) RIA kit (MP Biomedicals: #07138102). This antibody was raised against 6-keto-estradiol-17ß-oxime-BSA and has minor cross-reactivities with other estrogen metabolites (estrone: 20%; estriol: 1.5%; estradiol-17α: 0.7%). Samples were diluted (progesterone: 1:16 to 1:100; estrogen: 1:8 to 1:100) in standard zero buffer for analysis. All hormone values were reported as μg/g or ng/g feces.

For both assays, samples yielded dose-response curves parallel to the standard curve, and accuracy tests yielded recoveries of 104.1 ± 4.4% (N = 8) for progestins and 90.2 ± 10.0% (N = 6) for estrogens. Assay sensitivity was 31.25 pg/tube (6.25 ng/ml) for progestins and 0.125 pg/tube (5 pg/ml) for estrogens, calculated as the lower limit at which serial dilutions of the lowest standard no longer yield changes in hormone concentration. Intra-assay CVs for the high (20% binding) and low (80-85% binding) fecal pools were 2.8% and 7.2% for progestins and 6.2% and 1.7% for estrogens. Inter-assay CVs for high (20% binding) and low (80-85% binding) fecal pools were 9.9% and 16.3% for progestins and 6.0% and 10.6% for estrogens.

Analysis of Cycling Parameters

In primates, ovulation occurs in the period between the estrogen peak and the progesterone rise associated with the formation of corpus luteum (Lasley and Benirschke, 1994). The absolute time lag between a rise in circulating estrogens and ovulation is known to vary between hours and a full day (Nigi and Torii, 1983), while the lag between ovulation and the rise in luteal progesterone shows substantially more variation (hours to 2-3 days: Munro et al., 1991; Nigi and Torii, 1983). Hence, it is commonplace to use either the estrogen peak (e.g., Higham et al., 2008b; Yan and Jiang, 2006) or the beginning of the progesterone rise (e.g., Engelhardt et al., 2005; Heistermann et al., 1995) as a marker for ovulation. On the other hand, the component phases of the menstrual

cycle have more often been defined by patterns of progesterone, with the rise and fall in progesterone following ovulation marking the beginning and end of the luteal phase (e.g., Heistermann et al., 1995). However, it is well established that the periovulatory peak in estrogen levels is followed shortly by ovulation and the beginning of the luteal phase (see above). Furthermore, some studies have used the beginning of menses (Molskness et al., 2007; Shimizu et al., 2003) or the rise in estrogen levels from its postovulatory nadir (e.g., Emery and Whitten, 2003) to mark the beginning of the follicular phase.

We chose to use fE patterns rather than fP patterns to estimate the component phases of the cycle for two reasons: (1) fP elevations were not available for cycles during a period of plant-related elevation in fP levels (Fig. 2.1), and (2) analyses of the data showed that fP levels dropped to baseline within seven days of the rise, suggesting that the drop would have been an inadequate estimate of the end of the luteal phase (see results).

Because we occasionally had more than one sample per female per day, we used daily mean values of fE and fP levels to construct cycling profiles for all seven females sampled during Phase II. These profiles allowed us to identify 25 clear fE peaks, of which 24 were characterized by adequate sampling (every other day) near the day of the peak. For the one event occurring during a three day sampling gap, we estimated the day of the fE peak based on trends in the rise and fall of fE levels prior to and after the gap, using the fP values for confirmation. Of the total 25 fE peaks, nine occurred during a period of plant-related elevation of fP levels from mid-April 2006 to the end of the study period (Fig. 2.1, chapter 3). Because most of the remaining peaks (13 of 16) were followed, on average, within a day (range = 0-2 days) by a clear rise in fP levels 2.5 standard deviations above the previous baseline, we assumed ovulation occurs on the day of the estrogen peak.

However, there is usually a delay between hormone secretion into the bloodstream and the appearance of hormones in fecal matter due to gut passage and metabolism. This delay ranges, depending on diet and metabolism for each species, from hours to days (Bahr et al., 2000; Perez et al., 1988; Wasser et al., 1994). Because a previous study found that fecal glucocorticoids were excreted within 24 hours of an ACTH challenge in a related species (*Trachypithecus obscurus*: Lu and Czekala,

unpublished data), we assumed a 24-hour lag time between circulating hormones and their excreted counterparts. We thus identified the day prior to the fE peak as the ovulation date.

Note that our definition of ovulation assumes that estradiol peaks are sufficient to initiate ovulation. Estradiol rises during the follicular phase have been known to fail to induce a surge in luteinizing hormone, thus leading to an anovulatory cycle with no formation of the corpus luteum, and no correspondin rise in progesterone (Johnson, 2007). Often, this failure of ovulation is due to an insufficient estradiol rise (e.g., Dailey and Neill, 1981), or irregular estradiol patterns during the follicular phase (Johnson, 2007). In the present study, 13 of the 16 ovulations prior the high progesterin period were characterized by a fP rise following the fE peak. Although the remaining three were irregular, they were characterized by fP rises prior to and following the fE peak, suggesting some degree of corpus luteum function. And finally, for the nine cycles occurring during the high progestin period, five were conceptive, and the remaining four were characterized by fE peak levels (N = 4, range = 32.02 – 56.80 ng/g feces) well within the range of other cycles (N = 21, range = 25.89 – 100.89 ng/g feces). We are therefore confident that peak fE levels in the present study were sufficient to induce ovulation and can be used as an accurate marker for menstrual cycle parameters.

Hererin we refer to the day of the fE peak as Day 0. Thus, one day before the fE peak is Day -1, one day after the peak is Day 1, and so forth. All days were assigned post-hoc once hormone data were available. The luteal phase was calculated as the number of days from Day 1 (the estimated rise in fP, see above) to the day prior to the next fE rise from its postovulatory nadir (Fig. 2-2, see Emery and Whitten, 2003). We assumed that the rise in fE from its nadir (2 SD above preceding three values) indicates the beginning of follicular development. Similarly, the follicular phase was calculated as the first day of the fE rise, to the day of the next fE peak. Cycle length was defined as the time period from Day 0 (fE peak) until the next Day -1.

Defining Conception and Pregnancy

Conceptive cycles were defined as cycles where fE levels after ovulation exhibited a sustained increase above average follicular values (see also results). In such

cases, the conceptive date was defined as the ovulation date (Day -1) for that cycle. Gestation length was determined for the seven females sampled during Phase II, when frequent fecal sample collection allowed an accurate estimate of conception dates. Gestation length was estimated as the number of days from Day -1 to the day prior to the date of birth (documented retroactively from demographic records). In some cases, the group was not followed continuously, and the day of birth was estimated based on the median day of the period of time between the last group follow and the first day that the infant was seen. In these cases, minimum and maximum gestation lengths are also reported.

For changes in hormone levels across pregnancy, we focused on fE patterns because all pregnancies overlapped with the high progestin period. To evaluate fE patterns in the beginning of pregnancy, we used post-conceptive weekly hormone averages (N = 6-7 pregnancies). We then compared these values to both ovulatory peak fE levels during cycling and follicular mean fE levels prior to the peak. To examine variation in fE levels across pregnancy, we compared female means across trimesters. This dataset included four early pregnancies sampled across Phase I and II, in addition to the seven pregnancies sampled from only Phase II. Because conception dates were unavailable for the four pregnancies sampled earlier, trimester means were calculated backward based on the estimated parturition date and the estimated length of pregnancy (1^{st} trimester: wks -21 to -29; 2^{nd} trimester: wks -20 to -11; 3^{rd} trimester: wks -1 to -10). Because we only had complete pregnancies for four of the 11 females, weekly hormone profiles for the entire gestation period were constructed from a mixed longitudinal and cross-sectional dataset (N = 1-11 pregnancies per week, lower sample sizes later in gestation).

Statistics

All descriptive statistics assessed in the unit of days or cycles are reported with medians and ranges, as well means ± SEM. Statistical tests for cycling and pregnancy parameters were conducted using non-parametric analyses (SPSS Version 16; Sokal and Rohlf, 1995). Because sample sizes were small, differences in hormone values between trimesters were assessed using Kruskal Wallis and Mann Whitney U tests, treating

pregnancies, rather than females as the unit of analysis. Differences in fE levels between the initial weeks post-conception and cycling periods were assessed using Wilcoxon Signed Ranks Tests because adequate paired samples from the same female were available. Significance is reported at $\alpha = 0.05$ level. Bonferroni Holm corrections were made for all post-hoc analyses (Holm, 1979). Outlier analyses were conducted using Dixon's calculations (Dixon and Massey, 1969; Sokal and Rohlf, 1995).

RESULTS

Patterns of excretion of fE and fP for two of the seven females are shown from January 1 to September 28, 2006 (Fig. 2.3a-d). The hormonal profile for female B6 (Fig. 2.3a, 2.3b), who conceived prior to the high progestin period, was characterized by both fP and fE levels that show clear cycling patterns, with increasing levels of both hormones following conception. However, the profile for B9 (Fig 2.3c, 2.3d), who conceived during the high progestin period, was less easy to interpret. Specifically, fE and fP levels clearly reflect cycling patterns prior to the high progestin period, but the rise in fP (around mid-April) during the high progestin period obscured further patterns of cycling or conception. In contrast, fE patterns were still able to clearly define cycling and conception.

Cycling Patterns

We found that five of seven females experienced their first ovulations in January 2006 (Table 2.3). The remaining two females (B2 and B7) did not begin to ovulate until late February and late June, respectively. Based on the interval between successive fE peaks, female cycle lengths averaged 38.7 ± 4.1 days, with a frequency distribution skewed to the lower end of the range (Fig. 2.4; N = 18, median = 30, range = 21-90). On average, females experienced a minimum of 3.57 ± 0.43 cycles (N = 7, median = 3.0, range = 2-5) to conception.

Thirteen of the 16 cycles occurring prior to the high progestin period showed expected post-ovulatory increases in fP zero to two days following the fE peak (Fig. 2.5). However, fP levels remained elevated for only 7.0 ± 0.6 days (N = 10, median = 7.0, range = 3-10), suggesting that the fall in fP levels was an inadequate marker for the end

of the luteal phase (the luteal phase for other related species is around 11-15 days: He et al., 2001; Heistermann et al., 2004, Ziegler et al., 2000). Using fE patterns instead to estimate the component phases of the cycle, we found that the follicular phase was 25.7 ± 3.9 days long, with a median of 18.0 days (N = 18, range = 10-76). Correspondingly, the mean luteal phase was 12.2 ± 0.5 days with a median of 12.0 days (N = 18, range = 7-17). Follicular fE levels averaged 12.83 ± 1.26 ng/g feces, with levels prior to the peak averaging 10.19 ± 1.08 ng/g (N = 18), and peak levels averaging 48.05 ± 2.87 ng/g (N = 24). Average luteal phase fE levels were at 6.72 ± 0.53 ng/g feces (N = 18).

Since a previous study on baboons found that high progestin periods might influence female reproduction (Higham et al., 2007), we conducted a separate analysis of cycling parameters on data collected prior to the high progestin period only. After eliminating one outlier each for cycle length (value = 59 days, $r_{11} = 0.750$, $\alpha = 0.05$, Dixon's Outlier Analysis; Dixon and Massey, 1969; Sokal and Rohlf 1994) , and follicular phase length (value = 44 days, $r_{11} = 0.813$), we found that the cycle length during the normal progestin period was 28.4 ± 1.6 days (N = 10, median = 27.5 days, range = 21-40 days), with a follicular length of 15.4 ± 1.3 days (N = 10, median = 15.0 days, range = 12-26 days) and a luteal length of 12.5 ± 1.6 days (N = 12, median = 12.0, range = 11-17 days).

Three of the 16 cycles occurring during the normal progestin period showed irregular fP profiles (Fig. 2.6). Specifically each of the three showed a periovulatory rise in fP that was larger than the post-ovulatory rise. One particular ovulation occurred a few days following a major agonistic bout during which the female (B3) was the recipient of aggression from the adult male and several adult females in the group. During this event, she suffered from a major wound in the anogenital region. The two other irregular cycles were characterized by a minor peak in fE prior to the major peak (Fig. 2.6: B9, B12). Despite these differences, the luteal phases of all three (11-14 days) fell within the range of other cycles, and only one cycle was distinguished by a particularly long follicular phase (44 days) relative to the mean of 15 days.

Conception and Pregnancy

Most (5 of 6) conceptions occurred between late March (Mar. 21) and late June (Jun. 27) 2006, with the last conception occurring on September 7, 2006. Correspondingly, most (5 of 6) births took place between October 2006 and January 2007, with the latest female giving birth in early April 2007 (Table 2.4). From conception dates and birth records, we estimated a mean gestation length of 205.3 ± 1.41 days (N = 7, median = 204.0, range = 201-211, but see possible range, Table 2.4).

Conception and the beginning of pregnancy could be clearly distinguished by the last fE peak (Fig. 2.3b), following which fE dropped below periovulatory levels for one week before beginning its rise across pregnancy. Specifically, weekly fE levels were not significantly different (Fig. 2.7; N = 7, Wilcoxon Signed Ranks Test: $z = -2.366$, $p = 0.018$) from follicular levels (mean = 12.83 ± 1.26) until the second week of pregnancy (mean = 47.43 ± 2.65). Furthermore, although fE levels during weeks three to five of pregnancy exceeded that of the average fE peak for cycling females (N = 6, mean = 48.96 ± 2.37), fE levels did not remain permanently above the average fE peak until the eighth week of pregnancy (N = 6, mean = 79.94 ± 12.56; Wilcoxon Signed Ranks Test: $z = -2.201$, $p = 0.028$). Fecal estrogen levels were significantly different between trimesters (Kruskal Wallis Test: $H = 21.50$, $df = 2$, $p < 0.001$), increasing nearly 10-fold from the first (N = 11, mean = 59.94 ± 4.83) to second trimesters (N = 10, mean = 535.45 ± 72.64, Mann Whitney U Test: $z = -3.873$, $p < 0.0001$), with an additional two-fold increase from the second to third trimesters (Fig. 2.8; N = 6, mean = 1106.78 ± 132.31; Mann Whitney U Test: $z = -2.712$, $p = 0.007$). Post-parturition samples were only available from the four females sampled earlier (Phase I-early Phase II). We therefore did not have a large enough sample size to statistically evaluate differences between hormone levels during late pregnancy and those following parturition. However, data from these four pregnancies show that fE levels during the first three weeks following birth (Fig. 2.8; N = 4, Mean = 1.78 ± 0.41) clearly dropped from pregnancy levels and were no higher than average follicular fE levels (see above) calculated from the females in the subsequent year.

DISCUSSION

Here, we present the first results on the reproductive physiology in female Phayre's leaf monkeys, using non-invasive monitoring of steroid metabolites in a wild population. Our results suggest that both fE and fP excretion patterns provide useful information about the biology of this species. Across a female's menstrual cycle, fE levels, in particular, showed clear, consistent cycling peaks related to periovulatory estrogen surges (Fig. 2.2). Across pregnancy, fE levels exhibit: (1) a sustained increase from baseline to conception (Fig. 2.7), significant differences between each trimester throughout pregnancy, and a clear decrease at parturition (Fig. 2.8). However, because pregnancy fE levels did not increase significantly above periovulatory peak levels until the eighth week of gestation (Fig. 2.7), hormonal sampling during the first trimester only may not be adequate for accurate identification of pregnancy status.

Fecal progestin patterns also provided biologically relevant information on the reproductive physiology of Phayre's leaf monkeys. Although three cycles outside of the high progestin period were characterized by pre- and post-ovulatory increases in fP (Fig. 2.6), these cycles were also associated with either (1) a severe agonistic event or (2) the occurrence of a minor fE peak prior to the major one. Cycle irregularities due to stress are common in other species (e.g., Abbott, 1991; Heistermann et al., 2004) and may provide a potential explanation for the first case (Fig. 2.6, B3). However, the occurrence of both pre- and post-ovulatory fP rises, each associated with sharp increases in fE, are more difficult to explain and warrant further investigation. The occurrence of a pre-ovulatory fP rise in particular may suggest that relatively high amounts of progesterone can be secreted prior to the formation of the corpus luteum (see Lasley and Benirschke, 1994). Furthermore, the association of the fP rise with a small peak in fE suggests that this secretion may well be related to other events in the developing follicle.

For all other cycles prior to conception, fP patterns were able to distinguish the beginning of the luteal phase (Fig. 2.5) as well as the date of conception (Fig. 2.3a, 2.3b). However, fP patterns alone were inadequate for determining the end of the luteal phase (Fig. 2.2, 2.5). One possible explanation might be that *T. phayrei* excretes multiple progesterone metabolites, and that the antibody employed in the present study failed to detect one of the major ones associated with corpus luteum function toward the end of the

luteal phase. In Douc langurs (*Pygathrix nemaeus*: Heistermann et al., 2004) and long-tailed macaques (*Macaca fascicularis*: Heistermann et al., 2001a), high performance liquid chromatography (HPLC) studies have suggested that 5α-reduced 20-oxo pregnanes are major fecal metabolites of these species, indicating perhaps that a group-specific antibody may be more successful at capturing hormonal patterns of ovarian function in *T. phayrei* as well. Future work comparing multiple antibodies in relation to HPLC and/or radioinfusion studies (e.g., Wasser et al., 2000) might help resolve this matter.

The major impediment to using fP patterns to document reproductive function in this population was the occurrence of a seasonal increase in progesterone levels due to the consumption of phytoprogestins (chapter 3). Prior to this seasonal rise, fP levels showed clear biological patterns (e.g., Fig. 2.3a, 2.3b); however, fP levels after the rise showed no obvious pattern related to either cycling or conception (e.g., Fig. 2.3c, 2.3d). Similar patterns resulting from phytoprogestin consumption have recently been found in wild populations of chimpanzees (Thompson et al., 2008) and baboons (Higham et al., 2007) and suggest that the consumption of plant steroids may, at times, impede proper interpretation of reproductive state, conception, and cycle parameters from excreted fP metabolites alone.

Peak fE patterns suggest that first ovulations occurred in January (N = 5), with one female not ovulating until the end of February, and another not until June (Table 2.3). For all females except B3, we are confident that these ovulations represent the first cycles of the season because all samples collected 43-77 days prior to the first fE peak showed no rising trend. Female B3 had the oldest infant of all study females, (see Table 2.2), and could have begun cycling prior to the beginning of the intensive sample collection period in December 2005.

Thus, our reported average of 3.57 cycles to conception approximates, but most likely underestimates, the true number of cycles it takes for females to conceive. Females in this population thus take more cycles to conception than wild Hanuman langurs (1.88 cycles to conception: Ziegler et al., 2000). This is unsurprising, since Hanuman langurs are stricter in their birth seasonality (Koenig et al., 1997). In this respect, the number of cycles to conception in Phayre's leaf monkeys may be more similar to that of populations showing relaxed seasonal peaks (Alberts et al., 2005; Cheney et al., 2004), such as

baboons, which were reported to take between 2.2 and 4.7 cycles to conception (Beehner, 2003; Bentley-Condit and Smith, 1997; Smuts and Nicolson, 1989).

When we analyzed menstrual cycle parameters across the entire study period, we found an average cycle length of 38.7 days in duration, with a follicular length of 25.7 days, and a luteal length of 12.2 days. However, because the frequency distribution for cycle length was skewed towards the lower half of the range (Fig. 2.4), and because studies on both synthetic (e.g., Portugal and Asa, 1995) and plant-based progestins (e.g., Higham et al., 2007) have shown that elevated progestin levels can suppress reproduction in primates, we conducted a separate analysis of reproductive parameters during the normal progestin period only. After eliminating outliers, we found that the average cycle length was 28.4 days long, fitting well within the range reported for for Cercopithecoids (26 to 37 days: reviewed in Martin, 2007), but slightly longer in comparison with most studies on Colobines (22.4 - 26.4 days; He et al., 2001; Heistermann et al., 2004; Heistermann et al., 1995), even when studies in the wild are considered (22.4 - 27.0 days; Harris and Monfort, 2003; Ziegler et al., 2000). Note, however, that estimates based solely on behavior have found slightly higher values (27-30 days: Gibson and Chu, 1992; Ruempler, 1998). As in other studies (Heistermann et al., 2001b; van Schaik et al., 2000), the length of the follicular phase was more variable than that of luteal phase (Lasley and Benirschke, 1994), and extended cycle lengths were the result of elongated follicular phases. Indeed, while the mean luteal phase in the normal progestiner period (12.5 days) lies well within the range of previous colobine studies (11.5 – 14.8 days: He et al., 2001; Heistermann et al., 2004, Ziegler et al., 2000), the mean follicular phase, at 15.4 days was, again, slightly longer compared to previous studies (12.8 – 13.2 days: He et al., 2001; Heistermann et al., 2004, Ziegler et al., 2000). Factors such as female nutrition are known to impact primate ovulation (Ellison, 2003) and may have been an important variable influencing the longer cycles found even in the normal progestin period of the present study.

This study found a gestation length of 205.3 days for Phayre's leaf monkeys. In general, gestation length is a life history characteristic that scales with body mass (Martin, 2007). Based on the allometric relationship specific to primates, female Phayre's leaf monkeys, at a body mass of 6.3 kg (Smith and Jungers, 1997), are expected to have a

gestation length of around 180 days (e.g., Martin, 2007). The 205-day gestation length found in the present study is thus considerably higher than what is expected for primates in general. The same holds true when Phayre's leaf monkeys are compared to cercopithecines. For example, at 6.1 kg and 6.5 kg, respectively, *Macaca silenus* (Lindburg, 2001) and *M. nemestrina* (Hadidian and Bernstein, 1979) have estimated gestations lengths of 170 and 171 days.

Results for Phayre's leaf monkeys are, however, much more comparable to those reported for colobines. For example, *Trachypithecus cristatus* (Shelmidine et al., 2009) and *T. vetulus* (Rudran, 1973b) have gestation lengths of 194.6 and 197.6 days, and both weigh between 5.8 and 5.9 kg. Indeed, with the exception of *Colobus guereza* (158 days: Harris and Monfort, 2006), colobines in general seem to have longer gestations (~184 – 212 days: e.g., 184 days, *Colobus polykomos*: Dasilva, 1989; 200-212 days, *Semnopithecus entellus*: Sommer et al, 1992; Ziegler et al., 2000) compared to cercopithecines (~163-187 days: e.g., 163 days, *Macaca fascicularis*: Engelhardt et al., 2007; 187 days, *Papio ursinus*: Gilbert and Gillman, 1951), suggesting that there may be a phylogenetic, as well as an allometric influence on gestation length (Borries et al., in prep.).

DuFour and Sauther (2002) have argued that a longer pregnancy may increase the overall costs to gestation but decrease daily energetic costs. Because colobines are adapted to a folivorous diet (Chivers and Hladik, 1980), a prolonged gestation period may be a strategy to cope with sustaining pregnancy with the relatively low nutritious value of leaves. However, this hypothesis is inconsistent with data showing that colobine post-natal development is actually faster compared to other anthropoids (Leigh, 1994). Given these contradictory patterns, the role of diet in shaping colobine life history traits remains unresolved.

In conclusion, our study provides the first data on the reproductive characteristics of Phayre's leaf monkeys, using fecal steroid monitoring in a wild group. As such, we have extended our knowledge of the reproductive characteristics of cercopithecoids, particularly of the family *Colobinae* and the genus *Trachypithecus*. Our biological validation of steroid hormone measurement from feces and our general results on cycling

parameters, conception, and pregnancy provide the basis from which we can begin to examine further questions associated with female fertility and behavior.

Table 2.1 Relative age estimates for PB females. All females were multiparous at the start of the study.

Female	Relative Age
B2	Old
B3	Prime
B4	Old
B5	Prime
B6	Prime
B7	Young
B8	young
B9	young
B10	prime
B11	young
B12	prime

Table 2.2 Number of fecal samples and date of previous parturition for cycling females during the 2005-6 mating season.

		2005	2006									
Female	**Last Infant Born**	**Dec**	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**N Total**
B2	Apr 05	4	18	22	23	24	27	16	23	21	17	195
B3	Aug 04	6	20	22	22	27	25	27	27	25	23	224
B5	Mar 05	10	18	24	30	30	25	27	29	28	24	245
B6	Nov 04	20	25	35	27	28	28	22	19	25	15	244
B7	Dec 05							15	19	26	25	85
B9	Mar 05	8	18	25	25	29	26	28	28	22	18	227
B12	Feb 05	15	23	32	26	27	24	28	25	24	20	244

Table 2.3 Female cycle characteristics (x = no data, C = conceptive cycle, I = irregular cycle, HP = cycle occurring during the high progestin period from mid-April through September 2006). Range calculated when daily samples around markers for each phase were not available.

Female	Cycle	Cycle Notes	Ovulation Date	Follicular (days)	Luteal (days)	†Cycle length (days)
B2	1		2/28/2006	x	11	x
	2	C	3/21/2006	10	x	20-21
B3	1	I	1/9/2006	x	13-14	x
	2		2/18/2006	26-27	12-13	40
	3		*3/20/2006	16-17	17-18	28-32
	4	HP	5/6/2006	30-31	7-8	45-49
	5	C, HP	6/5/2006	23	x	30
B5	1		1/24/2006	x	13-15	X
	2		2/24/2006	16-17	12-13	29-32
	3		3/24/2006	15	12	27-29
	4	HP	4/18/2006	13	15-16	25
	5	C, HP	6/8/2006	35-36	x	51
B6	1		1/24/2006	x	13-15	X
	2		2/20/2006	12-13	12-13	27
	3	C	3/18/2006	13-14	x	25-27
B7	1	HP	6/22/2006	x	12	X
	2	HP	8/10/2006	37	11	48-50
	3	C, HP	9/7/2006	18	x	27-29
B9	1		1/29/2006	x	11-12	x
	2		2/28/2006	18	11-12	30-32
	3	I	3/27/2006	15	11-12	26-27
	4	HP	5/24/2006	46-47	x	58-59
B12	1		1/29/2006	x	13-15	X
	2	I	3/29/2006	44	14	58-60
	3	HP	6/27/2006	76	x	90-92

* Due to a three-day sampling gap at the time of the identified fE peak, ovulation date extrapolated from fE and fP patterns.
† Calculated from one fE peak to the day before the next fE peak (not from adding follicular and luteal phase lengths of each cycle).

Table 2.4 Previous parturition dates and gestation lengths of females.

Female	Estimated DOB	Length (days)	Possible Range (days)
B2	10/14/2006 – 10/16/2006	208	207-209
B3	12/25/2006	203	-
B5	12/29/2006 – 1/5/2007	208	204-211
B6	10/4/2006 – 10/6/2006	201	200-202
B7	4/1/2007 – 4/11/2007	211	206-216
B9	12/9/2006 – 12/20/2006	204	199-210
B12	1/12/2006 – 1/17/2006	202	199-204

(Note: Because it was not always possible to assess birth dates to the day, gestation length is calculated from the median of the range of possible birth dates.)

Figure 2.1. Mean daily fecal progestin values for non-pregnant females, showing the beginning and end of the high progestin period in 2006 (+ = mean daily fP (µg/g feces); error bars = ± SEM).

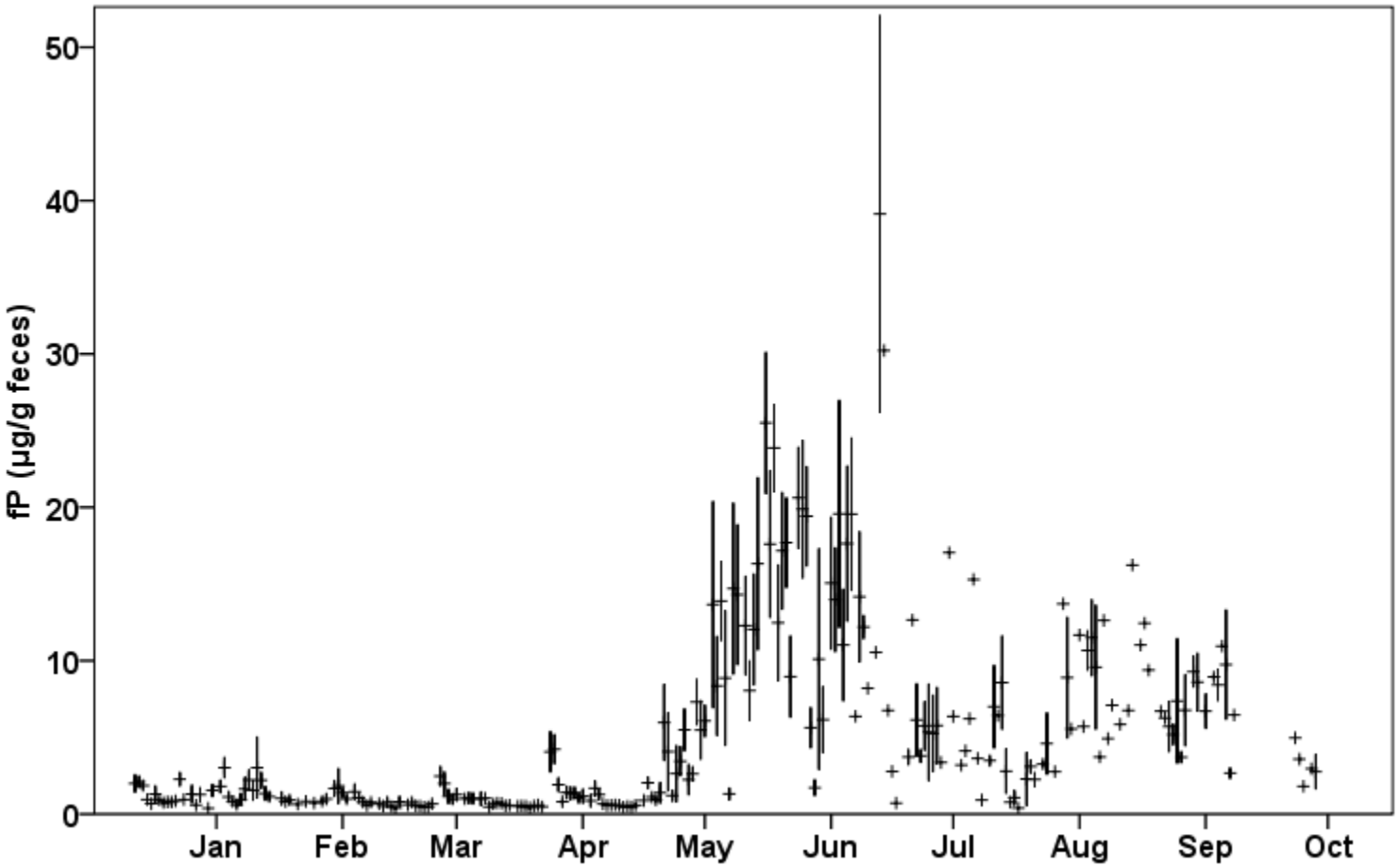


Figure 2.2. Example of luteal phase calculation from female B6 (January 13-February 22, 2006). Solid line = fE (pg/g feces); Dotted line = fP (µg/g feces); hatched area = luteal phase (calculated from the day after the fE peak to the day prior to the initial fE rise from its postovulatory nadir).

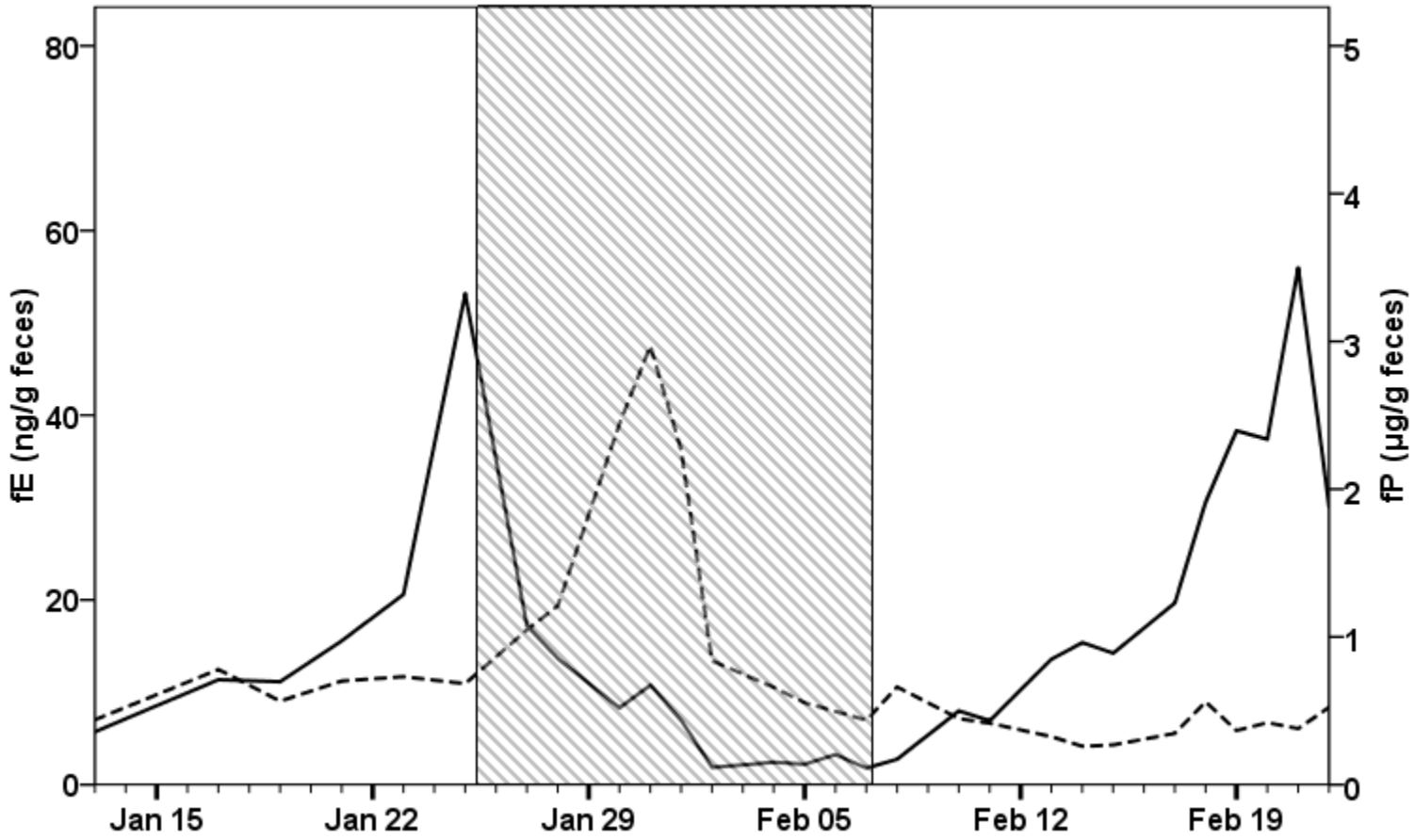


Figure 2.3a Fecal estrogen and progestin profile from cycling until late pregnancy for female B6, who conceived prior to the high progestin period. Solid line = fE (pg/g feces); Dotted line = fP (µg/g feces); arrow = conception date).

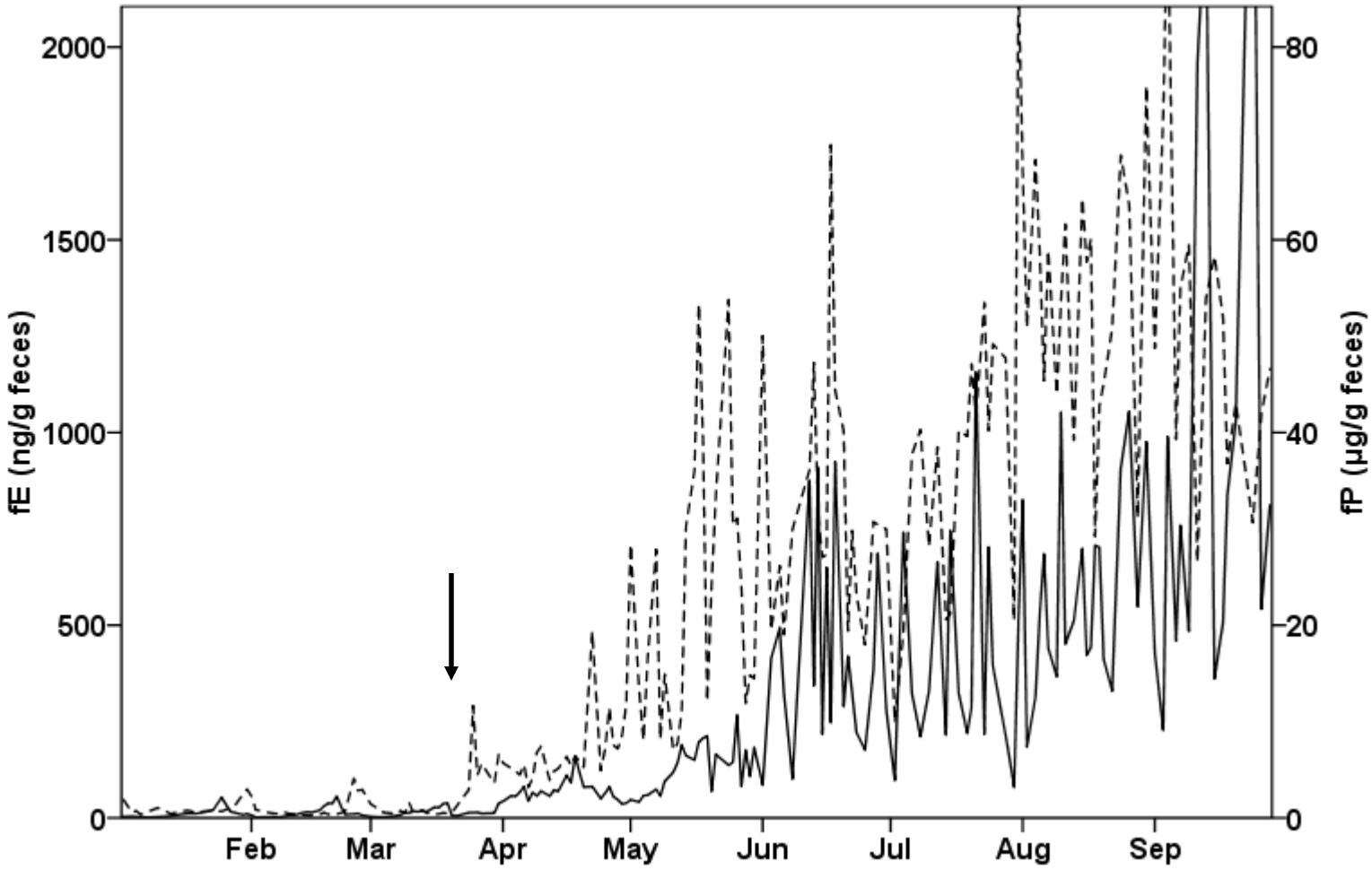
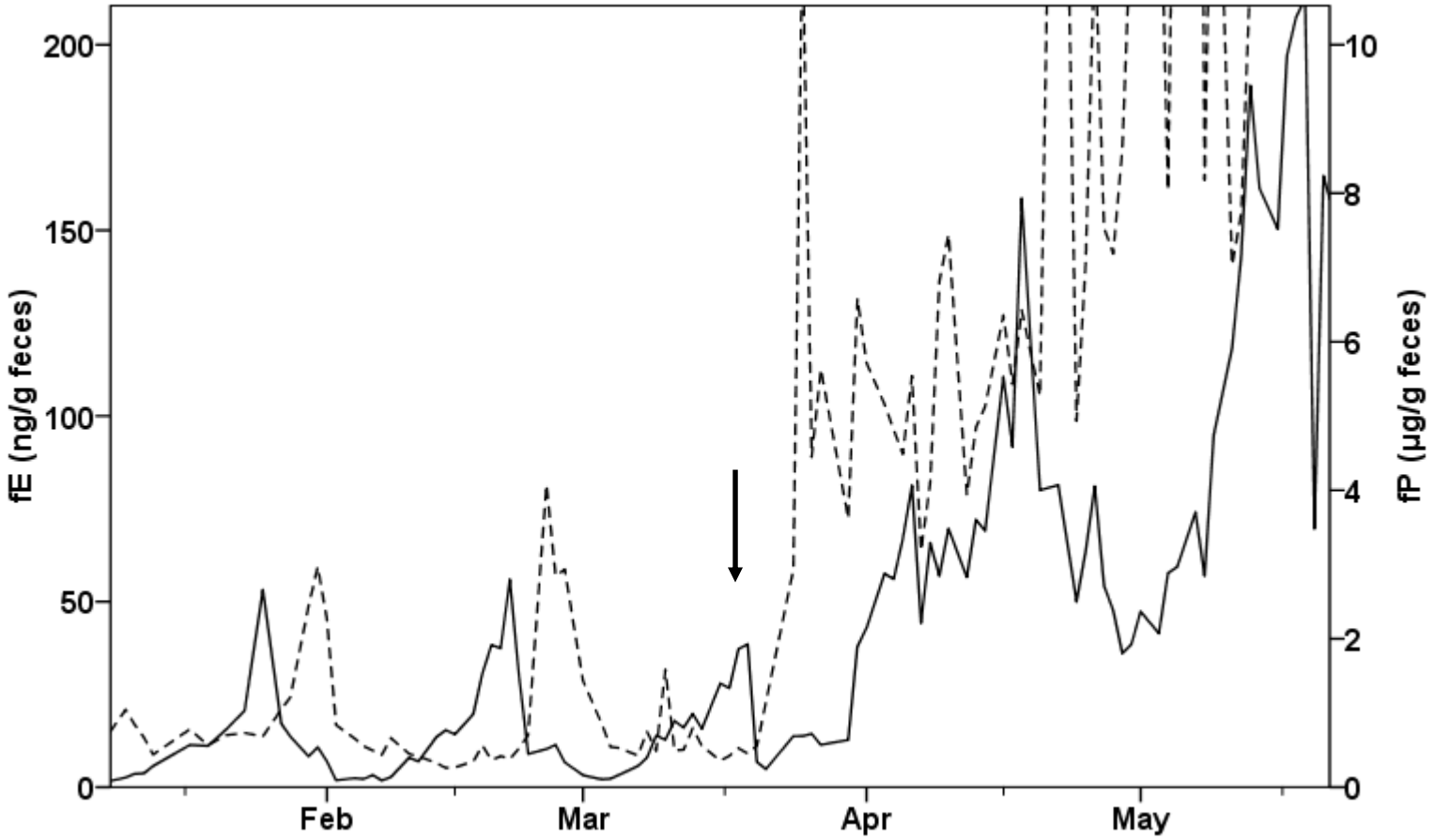


Figure 2.3b Fecal estrogen and progestin profile from cycling to conception for female B6, who conceived prior to the high progestin period. Solid line = fE (pg/g feces); Dotted line = fP (µg/g feces); arrow = conception date).



2.3

Figure 2.3c Fecal estrogen and progestin profile from cycling through the first four months of pregnancy for female B9, who conceived during the high progestin period. Solid line = fE (pg/g feces); Dotted line = fP (µg/g feces); arrow = conception date).

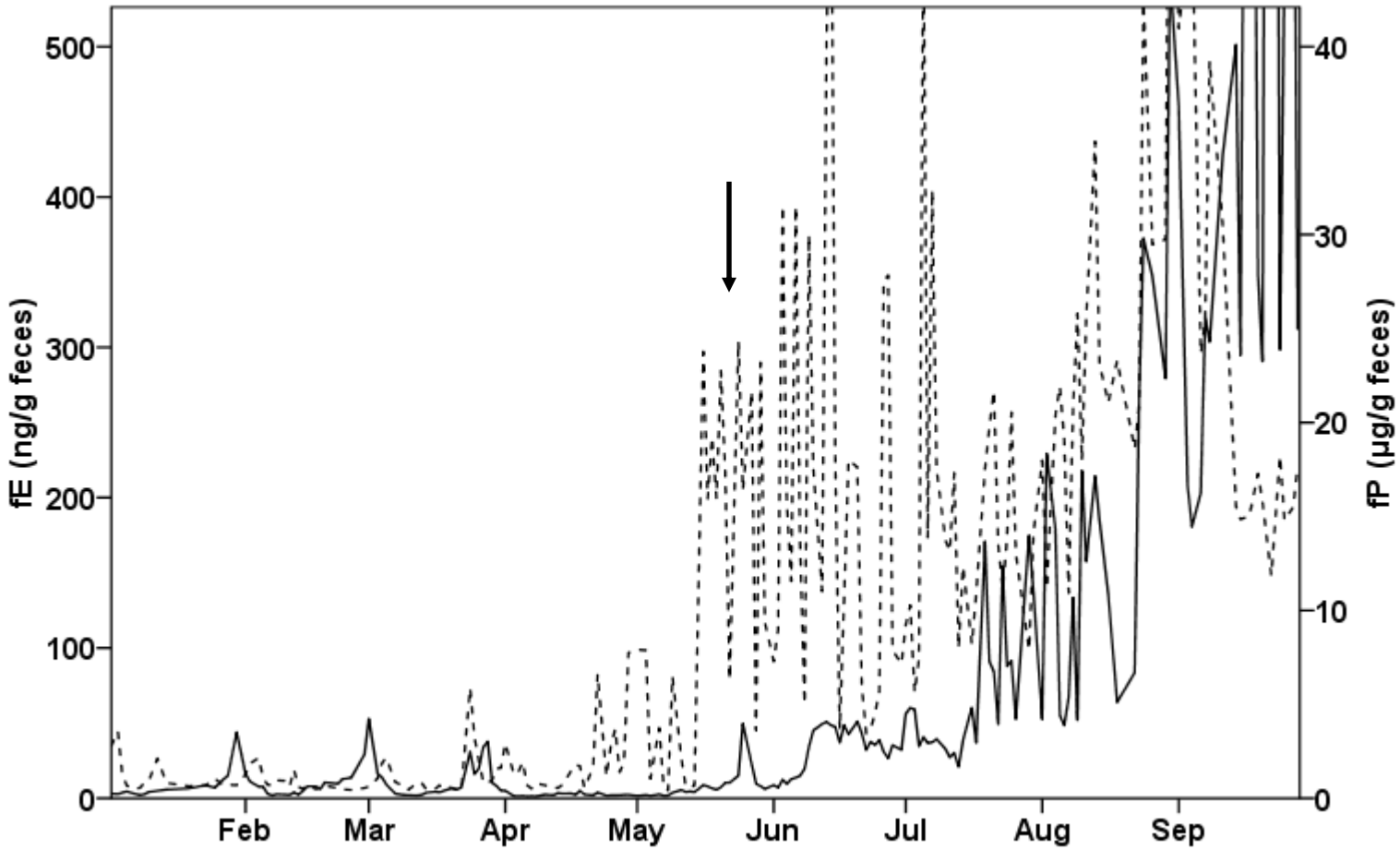


Figure 2.3d Fecal estrogen and progestin profile from cycling to conception for female B9, who conceived during the high progestin period. Solid line = fE (pg/g feces); Dotted line = fP (µg/g feces); arrow = conception date).

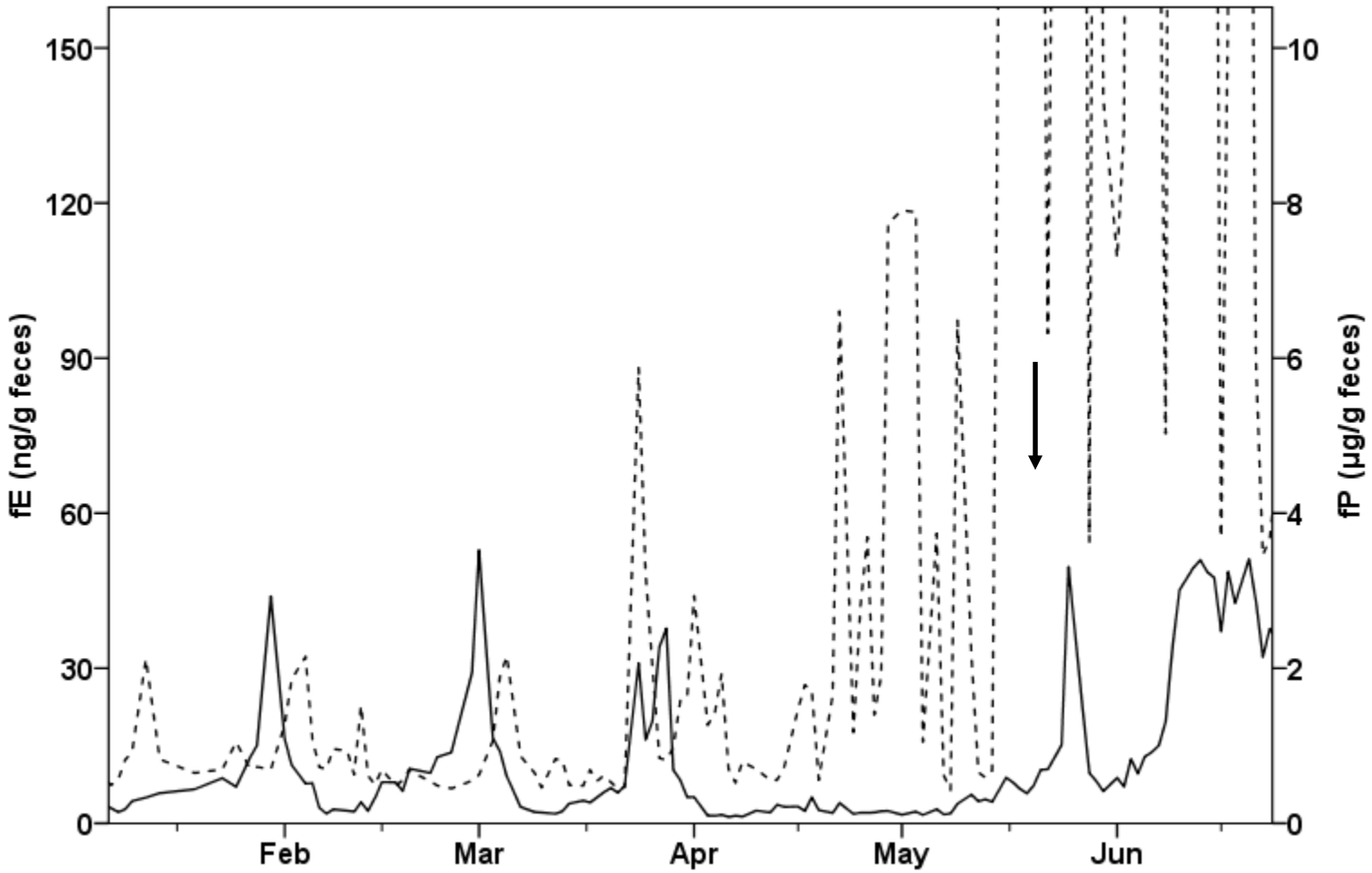


Figure 2.4 Frequency distribution of cycle lengths (N=18).

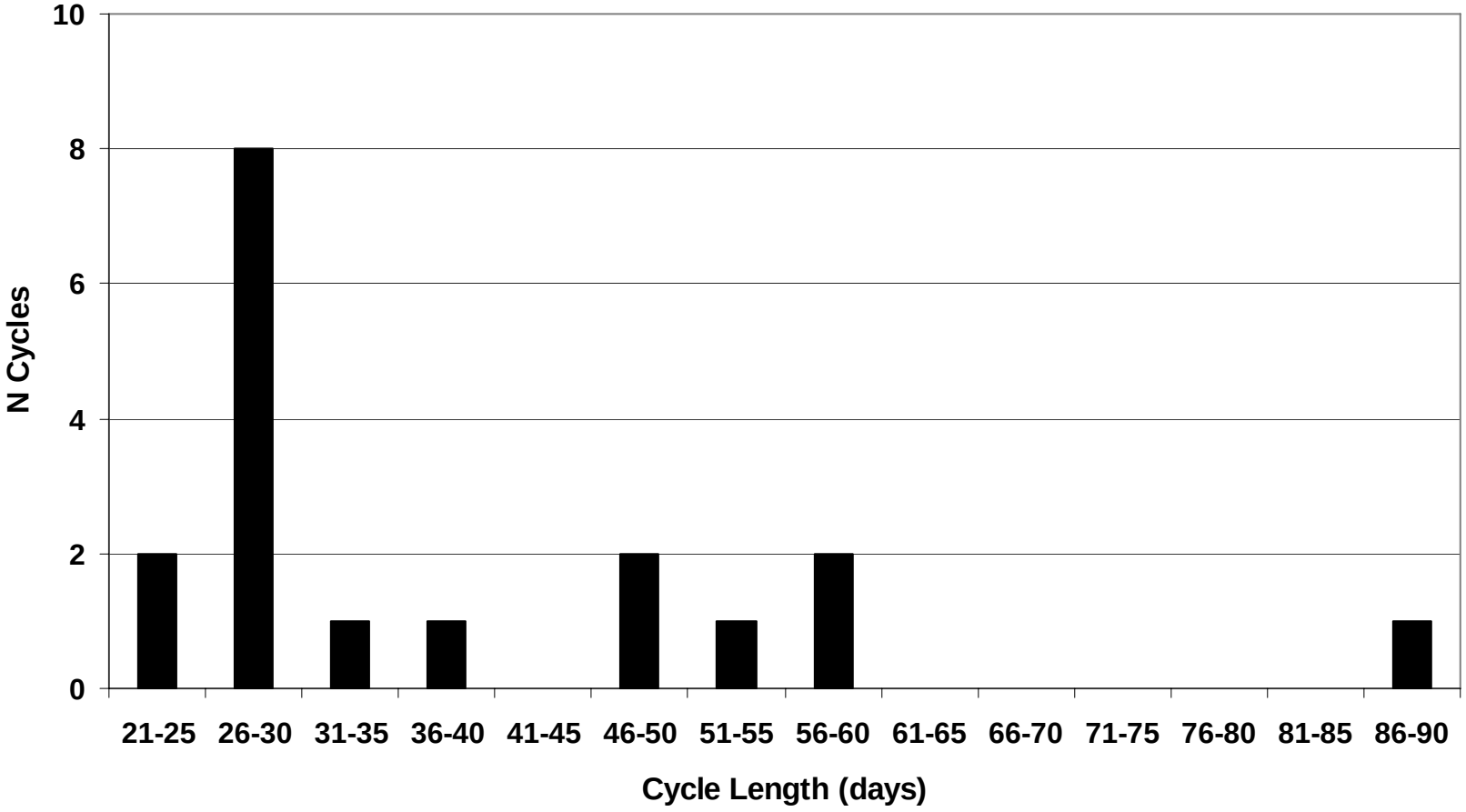


Figure 2.5 Composite menstrual cycle profile for 11 non-conceptive cycles (N = 6 females) occurring prior to the high progestin period. Solid line = fE (pg/g feces); Dotted line = fP (µg/g feces); error bars = ± SEM.

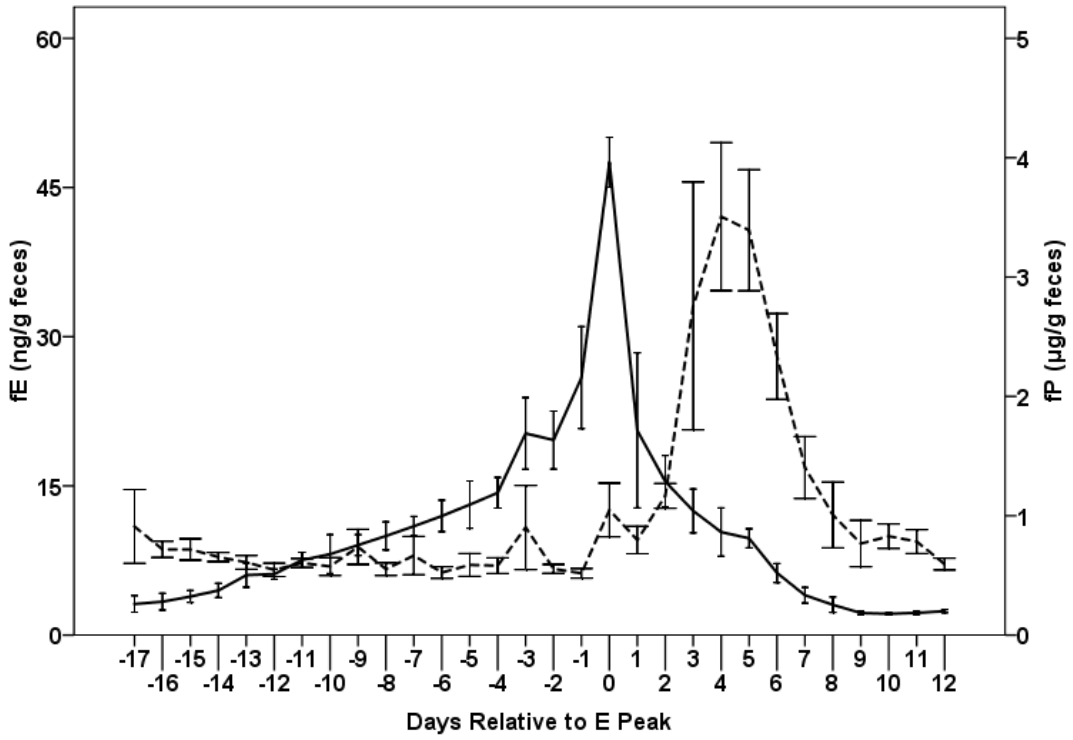


Figure 2.6 Irregular fecal hormone profiles for three cycles prior to the high progestin period from three different females B3, B9, and B12. Solid line = fE (pg/g feces); Dotted line = fP (µg/g feces).

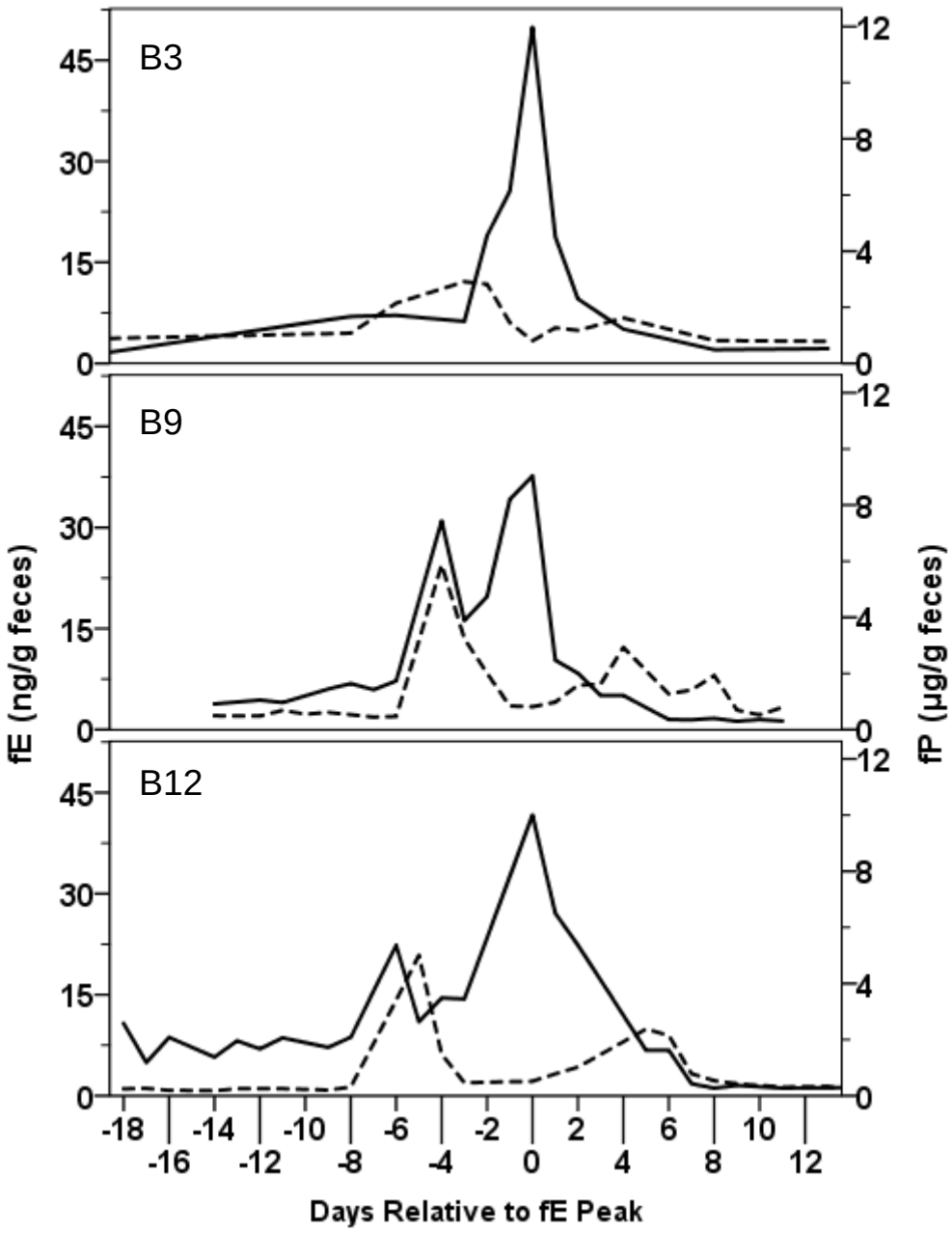


Figure 2.7 Composite fE (ng/g feces) profile from the two weeks prior to conception to the first 10 weeks of pregnancy (N = 7). Data are derived from a mixed longitudinal and cross-sectional dataset of seven pregnancies. Hatched area = mean ± SEM follicular fE levels; * = week at which fE levels are significantly different from follicular fE levels, ** = week at which fE levels remain statistically different from peak fE levels (Wilcoxon Signed Ranks Test, $\alpha = 0.05$).

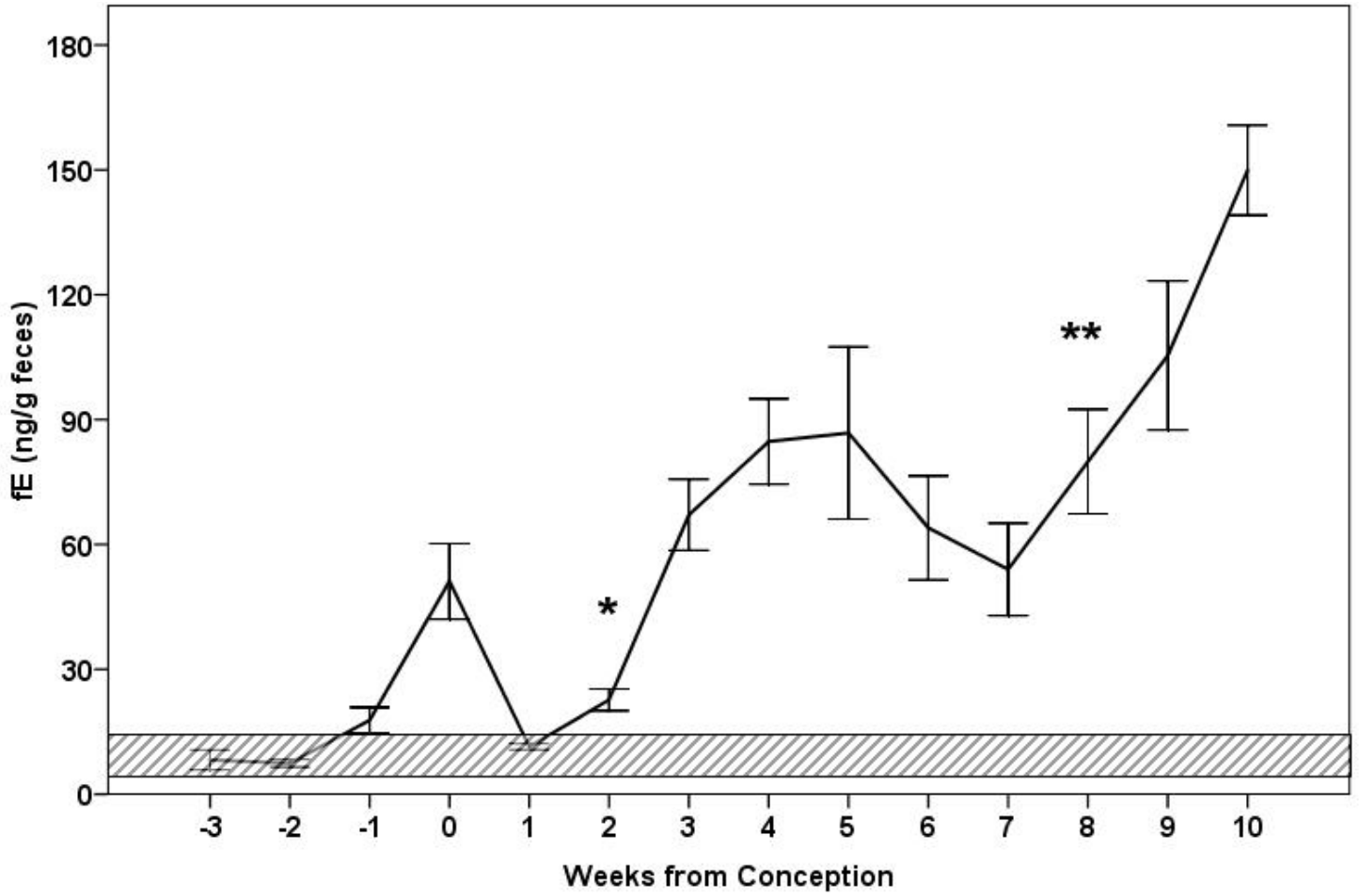
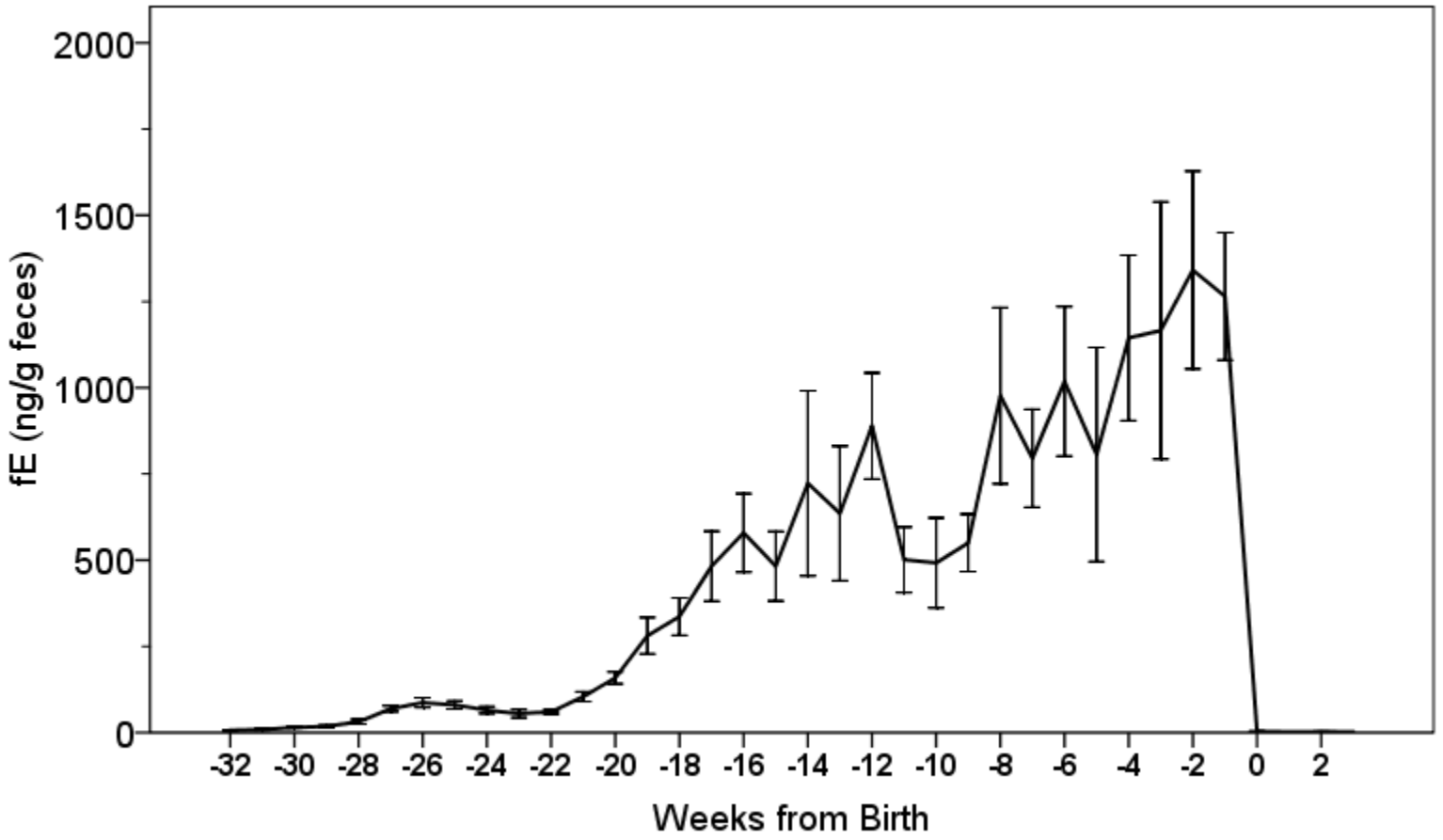


Figure 2.8 Composite fE (ng/g feces) profile for the entire pregnancy period (N=11 pregnancies). Data are derived from a mixed longitudinal and cross-sectonal dataset.



CHAPTER THREE

ECOLOGICAL VARIABLES AFFECTING REPRODUCTIVE FUNCTION

ABSTRACT

Studies on wild primate populations have suggested that the consumption of specific plant compounds, particularly from the genus *Vitex*, may influence female reproductive function via the effects of phytosteroids. Additionally, it is well established that food intake can affect reproduction via its influence on energetic status. Here, we present 20 months of data on seasonal fecal progestin (fP) patterns in wild female Phayre's leaf monkeys in Phu Khieo Wildlife Sanctuary. We examined whether *Vitex* availability might be associated with elevated fP levels by assessing the relationship between fP levels and the availability of *Vitex* leaves and fruits. In addition, we evaluated whether elevated progestins were associated with reproductive consequences, and whether these same reproductive consequences might also be consistent with changes in energetic status, as assessed by visual estimates of physical condition (PC). We collected fecal samples (N = 2046) from 10 females for the analysis of estrogen (fE) and progestin (fP) metabolites, behavioral data from cycling females (N = 7) for the analysis of female receptivity, and monthly data on *Vitex* availability and female PC. Seasonally elevated fP levels were found in all females over the course of two years, with patterns predicted by both reproductive condition (pregnant vs. non-pregnant) and the availability of *Vitex* leaves and fruits. High progestin periods were associated with significantly longer cycle lengths and follicular phases and with extended intervals between receptive periods. However, when ovulation occurred, conceptions were more likely during the high progestin period. Although these results support previous studies on the effects of *Vitex* and elevated progestins on reproductive function, they are also consistent with changes in female PC, ie., increasing PC was associated with a greater probability of conception; and decreasing PC was associated with delayed ovulation (but only during the high fP period). Ultimately, the effects of phytosteroids and nutritional status may be difficult to separate. Nevertheless, because plants with phytosteroid compounds are common to the

diet of many primate populations, studies on the chemical composition and function of such plants might help us better explain reproductive patterns in the wild.

INTRODUCTION

The idea that specific plant compounds might influence reproduction is not new to behavioral ecologists. Studies on wild primate populations, for example, have suggested that the consumption of specific food items might influence the timing of sexual behavior and conception (e.g., Wallis, 1997; Whitten, 1983) as well as the sex of the infant (Glander, 1994). Researchers have often attributed these reproductive effects to the action of phytosteroids (e.g., Garey, 1991; Garey et al., 1992; Wallis, 1997; Whitten, 1983).

Although mammalian steroids such as estrogens, progesterone, and various androgens have been identified in many plant species (Iino et al., 2007; Janeczko and Skoczowski, 2005), they are usually too low in concentration to affect behavior (Fritsche and Steinhart, 1999). Phytosteroids, in contrast, are plant compounds that may or may not be structurally identical to endogenous steroids but influence their function by competing for steroid binding sites, altering steroid biosynthesis or metabolism, or interfering with steroid transport (Whitten and Naftolin, 1998; Whitten and Patisaul, 2001; also see Clotfelter et al., 2004). They may mimic the function of mammalian steroids, but they have the potential to exhibit both agonistic and antagonistic effects. The direction of such effects often depends on the type or dosage of the plant compound involved (Whitten and Naftolin, 1998; Whitten and Patisaul, 2001). To date, most phytosteroids have been identified by their interference with endogenous estrogens, primarily via competition for estrogen binding sites. However, plant extracts have also been shown to interfere with the action of other hormones, such as progesterone, androgens, and prolactin (e.g., Beck et al., 2003; Benie and Thieulant, 2003; Wuttke et al., 2003).

Unsurprisingly, there is abundant evidence that phytosteroids can interfere with reproductive function. For example, diets rich in phytoestrogens lead to deficits in sexual development in male and female Japanese quail (*Coturnix japonica*: Rochester et al., 2009) and rainbow trout (*Oncorhynchus mykiss*: Bennetau-Pelissero et al., 2002). In California quail hens (*Callipepla californica*: Leopold et al., 1976), ingestion of phytoestrogens from the leaves of subclovers reduced egg-laying rates, and in female ruminants, consumption of clover- and alfafa-based phytoestrogens reduced ovulation and conception rates (Adams, 1995). Similar effects have been demonstrated in captive studies on non-human primates. Studies on long-tailed macaques (*Macaca fascicularis*)

have shown that plant extracts from a Thai herb, *Puerararia mirifica*, decrease gonadotropin levels (Trisomboon et al., 2006b), and cause both a reddening of the sexual skin around the perineum (Trisomboon et al., 2006a) and an extension of the length of the follicular cycle (Trisomboon et al., 2004; 2005). Finally, studies on vervets (*Chlorocebus aethiops*) have found that diets supplemented with extracts from *Acacia elatior* cause an increase in urinary progestins (Garey, 1991; Garey et al., 1992).

More recently, data from wild primates have shown that the consumption of fruits from the genus *Vitex* (*V. doniana, V. fischeri*) led to increased levels of excreted progestin metabolites in baboons (*Papio anubis*: Higham et al., 2007) and chimpanzees (*Pan troglodytes*: Thompson et al., 2008), in the range comparable to, or even above, that found during pregnancy. In baboons, the elevated period of progestins was also associated with a lack of sexual swelling, suggesting that chemical compounds found in at least one *Vitex* species (*V. doniana*) can directly impact sexual function: in this case, sexual attractivity. Furthermore, Higham et al. (2007) suggested that elevated progestin levels due to *Vitex* consumption may function in the same way as progestin-based contraceptives. That is, they mimic pregnancy, prevent ovulation, and suppress sexual signaling/behavior. Indeed, synthetic progestins are reported to inhibit ovarian function in females and sexual function in both males and females in many species of primates (*Macaca arctoides*: Steklis et al., 1982; *M. fascicularis*: Michael and Zumpe, 1993; Pazol et al., 2004, Shimizu et al., 1996, Zumpe et al., 1997; Zumpe et al., 2001; *Papio hamadryas*: Portugal and Asa, 1995; *Pan troglodytes*: Bettinger et al., 1997). Further support for *Vitex doniana* as a contraceptive comes from traditional practices whereby a related species, *Vitex agnus castus* ("chasteberry"), has been prescribed for the control of libido in human males (Roemheld-Hamm, 2005).

In terms of conception, however, human studies have shown that *V. agnus castus* extract can treat luteal phase dysfunction by increasing luteal phase progesterone levels, and thus increasing the chances of conception (Gerhard et al., 1998; Milewicz et al., 1993; Westphal et al., 2004). It is well known that elevated progesterone levels are necessary for successful conception – a necessity exploited by the use of RU486 ("the morning after pill"), a synthetic contraceptive that acts as a progesterone antagonist by competing for binding with progesterone receptors (Mahajan and London, 1997, but see

Landgren, 1990 for other synthetic progestins). Therefore, the effects of *Vitex* on female reproductive function seem to indicate a dual effect: While elevated progestin levels due to *Vitex* spp. consumption might act as a contraceptive that impairs ovulation, if ovulation *does* occur, it may enhance the chances of conception.

Given that primate reproduction is often seasonal and associated with both general and specific availability of plant foods, the aforementioned studies on the effects of phytosteroids suggest that the chemical constituents of plant products might also play a role in shaping the timing of reproduction and conception in female primates. However, food consumption itself is also more generally associated with reproduction via its effects on overall energetic condition (Bronson, 1985; Ellison, 2003). Studies on humans and nonhuman primates have shown that the likelihood of ovulation (e.g., Ellison, 1989; Frisch and McArthur, 1974; Maninger et al., 2000; Walker et al., 1983) and conception (e.g., Ellison, 1989; Koenig and Borries, 2001; Koenig et al., 1997; Thompson and Wrangham, 2008; van Schaik and van Noordwijk, 1985) are sensitive to either current energetic status or changing energetic status (flux), and suggest that temporal changes in food availability, feeding, and/or energy expenditure directly shapes seasonality in reproduction (reviewed in Brockman and van Schaik, 2005; Bronson, 1985; Ellison et al., 2005). Hence, the consumption of food resources may affect female reproduction simply via its effects on female energetic status, independent of the effects of phytosteroids (see Knott, 2001).

In the current study, we examine the effect of phytosteroids and nutritional status on female reproduction in wild Phayre's leaf monkeys (*Trachypithecus phayrei crepusculus*). Specifically, we (1) investigate whether fecal progestin (fP) metabolites exhibit seasonality, independent of changes in reproductive status, (2) examine whether seasonal patterns might be related to *Vitex* consumption, using data on the availability of *Vitex* spp. as an indirect test, and (3) assess the effect of both elevated fP levels and energetic condition on several measures of reproductive function (e.g., receptivity, cycle length, and the probability of conception). In relation to reproduction, we predict specifically that seasonal periods of elevated fP will reduce the probability of ovulation and decrease sexual receptivity, therefore leading to longer cycle lengths and follicular phases, shorter receptive periods, and longer inter-mating intervals. In relation to

conception, we test the hypothesis that elevated phytoprogestins, as indicated by seasonal periods of high fP levels, may increase the probability of conception should ovulation occur. Finally, we predict that these same effects – specifically, a reduced probability of ovulation and increased probability of conception – might be related to increasing and decreasing physical condition.

METHODS

Study Site

The study was conducted in Phu Khieo Wildlife Sanctuary, located in northeast Thailand (16°5‘- 35‘N, 101°20‘-55‘E, Chaiyaphum Province, elevation: 300-1300 m above sea level). Encompassing an area of 1,573 km^2, the sanctuary is home to a diverse group of vertebrates, including seven primate species (Borries et al., 2002), eight species of felids, 10 viverrid species, and two raptor species (Grassman et al., 2005). The study site of Huai Mai Sot Yai is located at 16°27'N, 101°38'E, at an elevation of 600-800 m. The forest consists of dry evergreen, with patches of dry dipterocarp (Borries et al., 2002). Rainfall averages 1,400 mm per year. Although rain can occur during all months, the majority falls in the second half of the rainy season between June and October (Grassman et al., 2005; Kumsuk et al., 1999). In contrast, very little rain occurs in December and January, and peaks in rainfall during the remaining months can still vary dramatically from year to year (Fig. 3.1). Other than a few legume species that are available during the dry season, fruiting patterns follow the pattern of rain, with months of abundant food coinciding with peaks in the rainy season (Koenig and Borries, unpublished data).

Subjects & Study Period

Research took place as part of an ongoing study conducted by Andreas Koenig and Carola Borries since October, 2000. The current study was carried out between February 1, 2005 and September 28, 2006 (20 months), focusing on one group called PB. This group was habituated since spring of 2004, and demographic data have been collected since June, 2003 (Borries and Koenig, unpublished data). At the beginning of the study period, PB consisted of one adult male, 11 adult females, and 12 immatures.

Births (7), disappearances (3), and emigration of one adult female (Borries and Koenig, unpublished data) changed the composition of the group during the course of the study. In addition, two juvenile males matured and became subadults in 2006. By the end of the study, the group contained the same adult male but had two subadult males, 10 adult females and 14 immatures.

Our study was conducted in two phases: (1) During Phase I (02/01/05 – 12/12/05) all adult females (N = 11) were followed 12-20 days per month (147 (53.5%) of 275 days, 1662.9 contact hours, 11.3 hours/day), with monthly collection of fecal samples for broad estimates of reproductive state. (2) During Phase II (12/13/05 – 9/28/06), group follows were more frequent to collect fecal samples for estimates of ovulation and conception (265 (90.7%) of days; 3058.9 contact hours, mean = 11.5 hours/day) and specifically targeted cycling females (N = 7). We determined the onset of Phase II based on birth peaks from previous years, a 200-day estimated gestation length (Rudran, 1973a; Ziegler et al., 2000), and a conservative estimate of three cycles to conception (Dixson, 1998; see chapter 2 for details). Initial identification of cycling females (N = 5) was based on a two-year interval after surviving infants (Borries et al., 2007). Additional females (N = 2, totaling 7) were added to the sample because they were observed to mate (see chapter 2).

In this population, reproduction is not strictly seasonal. While births can occur during all months of the year, the majority take place between November and April, suggesting a conception peak between May and October, coinciding with the latter half of the rainy season. Dietary and rainfall data suggest that conceptions are timed during energetically favorable periods of the year (Borries and Koenig, unpublished data; Borries et al., 2005).

Fecal Sample Collection

A total of 2046 samples were collected from 10 females for the analysis of fecal steroids. Samples from an additional female (B8) were not included in the study because she immigrated shortly after the study commenced (May 2005). During Phase I, 387 samples were collected from all 10 adult females on a monthly basis (1-7 samples/month). The majority of samples (N = 1,659) were collected during Phase II

(Dec 13, 2005 – Sep 28, 2006). During this period, cycling females were targeted every other day for sample collection, while all other females were sampled as in Phase I. For all complete months of data collection in the second phase, fecal samples were collected on an average of 22.3 days/female/month for cycling females.

Samples were collected in 30 ml plastic vials (Sarstedt: vial #75.1337.500, lid #76.1340.560), kept on ice in the field, and frozen (-20°C) upon return to the field station (within 2-13 hrs). In December 2006, samples were shipped on ice to the Conservation and Research of Endangered Species (CRES) at the Zoological Society of San Diego.

Fecal Hormone Analysis

At CRES samples were lyophilized and sifted through mesh wire (16 x 16 mesh; McMaster-Car: # 9223T82) to remove vegetative matter. Samples were then transported to the Core Assay Facility in the Department of Psychology at the University of Michigan for further analyses. At the University of Michigan, samples were doubly extracted following protocols established by Wasser et al. (1994). Extracted samples were reconstituted in 100% ethanol, transferred into 2ml microcentrifuge tubes (Sarstedt: #72.694.007) and stored in the freezer (-20°C) until analysis. Extraction of radiolabeled progesterone and estrogen yielded recoveries of 86.7 ± 3.8% (N = 10) and 91.5 ± 0.8% (N = 10), respectively.

Progesterone and estrogen metabolites were analyzed using radioimmunoassay (RIA) (chapter 2 for details). For both assays, samples yielded dose-response curves parallel to the standard curve, and accuracy tests yielded recoveries of 104.1 ± 4.4% (N = 8) for progestins and 90.2 ± 10.0% (N = 6) for estrogens. Parallelism for the progestin assay was documented during both the normal and the high progestin period. Assay sensitivity was 31.25 pg/tube (6.25 ng/ml) for progestins and 0.125 pg/tube (5 pg/ml) for estrogens, calculated as the lower limit at which serial dilutions of the lowest standard no longer yield changes in hormone concentration. Intra-assay CVs for the high (20% binding) and low (80-85% binding) fecal pools were 2.8% and 7.2% for progestins and 6.2% and 1.7% for estrogens. Inter-assay CVs for high (20% binding) and low (80-85% binding) fecal pools were 9.9% and 16.3% for progestins and 6.0% and 10.6% for estrogens.

Cycle Characteristics and Definitions of Reproductive State

Previous studies have used either the estrogen peak (e.g., Higham et al., 2008b; Yan and Jiang, 2006), or the beginning of the progesterone rise (e.g., Engelhardt et al., 2005; Heistermann et al., 1995) as a marker for ovulation. On the other hand, the component phases of the menstrual cycle have more often been defined by patterns of progesterone, with the rise and fall in progesterone following ovulation marking the beginning and end of the luteal phase (Heistermann et al., 1995). However, it is well established that the periovulatory peak in estrogen levels is followed shortly by ovulation and the beginning of the luteal phase (Lasley and Benirschke, 1994). Furthermore, some studies have used the beginning of menses (Molskness et al., 2007; Shimizu et al., 2003) or the rise in estrogen levels from its postovulatory nadir (e.g., Emery and Whitten, 2003) to mark the beginning of the follicular phase. Because cycling patterns for fP were absent during the portion of the study when fP levels were seasonally elevated (see results, also chapter 2), we used fE patterns instead to assess cycle parameters (i.e. ovulations, cycle length, length of the luteal and follicular phases).

We occasionally had more than one sample per female per day. Therefore, we used daily mean values of fE and fP levels to construct cycling profiles for all seven females sampled during Phase II. These profiles allowed us to identify 25 clear fE peaks, of which 24 were characterized by adequate sampling (every other day) near the day of the peak. For the one event occurring during a three day sampling gap, we estimated the day of the fE peak based on trends in the rise and fall of fE levels prior to and after the gap, using the fP values for confirmation. Of the total 25 fE peaks, nine occurred during the period of seasonally elevated fP levels from mid-April 2006 to the end of the study period. Because most of the remaining peaks (13 of 16) were followed, on average, within a day (range = 0-2 days) by a clear rise in fP levels 2.5 standard deviations above the previous baseline, we assumed ovulation occurs on the day of the estrogen peak. A previous study found that fecal glucocorticoids were excreted within 24 hours of an ACTH challenge in a related species (*Trachypithecus obscurus*: Lu and Czekala, unpublished data). We therefore assumed a 24-hour lag time between circulating

hormones and their excreted counterparts. We thus identified the day prior to the fE peak as the ovulation date.

Note that our definition of ovulation assumes that estradiol peaks are sufficient to initiate ovulation. Estradiol rises during the follicular phase have been known to fail to induce a surge in luteinizing hormone, thus leading to an anovulatory cycle with no formation of the corpus luteum, and no correspondin rise in progesterone (Johnson, 2007). Often, this failure of ovulation is due to an insufficient estradiol rise (e.g., Dailey and Neill, 1981), or irregular estradiol patterns during the follicular phase (Johnson, 2007). In the present study, 13 of the 16 ovulations prior the high progesterin period were characterized by a fP rise following the fE peak. Although the remaining three were irregular, they were characterized by fP rises prior to and following the fE peak, suggesting some degree of corpus luteum function. And finally, for the nine cycles occurring during the high progestin period, five were conceptive, and the remaining four were characterized by fE peak levels (N = 4, range = 32.02 – 56.80 ng/g feces) well within the range of other cycles (N = 21, range = 25.89 – 100.89 ng/g feces). We are therefore confident that peak fE levels in the present study were sufficient to induce ovulation and can be used as an accurate marker for menstrual cycle parameters.

Hererin we refer to the day of the fE peak as Day O. Thus, one day before the fE peak is Day -1, one day after the peak is Day 1, and so forth. All days were assigned post-hoc once hormone data were available. The luteal phase was calculated as the number of days from Day 1 (the estimated rise in fP, see above) to the day prior to the next fE rise from its postovulatory nadir (see chapter 2, Fig. 2-2; Emery and Whitten, 2003). We assumed that the rise in fE from its nadir (2 SD above preceding three values) indicates the beginning of follicular development. Similarly, the follicular phase was calculated as the first day of the fE rise, to the day of the next fE peak. Cycle length was defined as the time period from Day 0 (fE peak) until the next Day -1. Conceptive cycles were defined as cycles where fE levels after ovulation exhibited a sustained increase above average follicular values (see chapter 2). In such cases, the conceptive date was defined as the ovulation date (Day -1) for that cycle.

In Phayre's leaf monkeys, infants are often in nipple contact while adult females return to cycling, often remaining in nipple contact up until the birth of the subsequent

infant. In this paper, we thus refer only to "cycling" when females show fE profiles indicative of follicular development and ovulation. Because we did not have adequate samples from Phase I to distinguish cycling females from females that were only lactating, we also defined reproductive state broadly for all females, distinguishing pregnant and non-pregnant states. Pregnancy was determined for Phase II females via fE patterns. For all earlier pregnancies, we used a combination of fE patterns, coupled with estimates of conception based on the average gestation length (205 days) of females in this group (chapter 2).

Behavioral Data Collection & Definition of Receptive Periods

Through the study period, focal and *ad libitum* data (Altmann, 1974) on sexual behavior were collected by assistants and A.L. Focal follows were conducted on all adult females in a pre-determined, random order, with each lasting for 20 minutes. During these follows, we collected continuous data on attractive, proceptive, and receptive behavior (Beach, 1976). *Ad libitum* data on sexual behavior were collected during focal follows, as well as during follows of cycling females for the collection of fecal samples. For the purposes of this paper, we only analyzed receptive behavior. These included copulations (intromissions), as well as attempted copulations (mounting without intromission) (Sommer et al., 1992).

For behavioral analysis of receptive periods, we included data collected from the last eight months of the study (February 1 to September 28, 2006), when follows occurred almost daily (220 of 240 days; 2603 group hours) and all observers (AL and two assistants) were appropriately trained in identifying individual monkeys and collecting data on sexual behavior. Following previous studies (Borries et al., 2001; Shelmidine et al., 2009; Sommer et al., 1992), receptive periods were defined as a set of consecutive days (not interrupted by more than two days) during which a female copulated or attempted to copulate. A receptive period could be one day long only when at least one copulation was observed. The inter-mating interval was then calculated as the number of days between the first day after one receptive period to the last day before the next receptive period.

Phenology

With the exception of the first two months of the study period, phenological data were collected once per month, usually in the middle of the month, over the course of four to six days. Initially, the overall phenology sample consisted of 480 trees and climbers belonging to 110 species. The sample was later (August 2006) extended to include 546 trees and climbers.

There were a total of four different *Vitex* species (*V. glabrata, V. penduncularis, V. quinata, V. scabra*) identified at the study site; however, because they were generally rare in the plots (<5 trees in all botanical plots), they were only represented by 1-2 trees per species in our phenology sample. Over the course of the study, PB was observed to feed on the genus *Vitex*, focusing mostly on *V. scabra* and *V. quinata* species. Because of the scarcity of tree samples in the plots however, and because *Vitex* phenophases usually occurred at similar times, we combined all *Vitex* species (N = 6 total trees) for analyses.

Phenology was assessed on a $\log_{10}$ scale (i.e., 0 for 1-9 parts, 1 for 10-99 parts, etc.; Janson and Chapman, 1999), for each of the following phenophases: leaf bud, young leaf, mature leaf, flower bud, full flower, young fruit, mature fruit, old fruit. We ensured the continuity of data assessments by having all data collected by teams of two people, one of whom had long-term experience with the data collection protocol. Since our study group was observed to feed predominantly on young leaves and young and mature fruit of *Vitex* species, we included these three phenophases in our analyses.

Physical Condition

Following previous studies (Berman and Schwartz, 1988; Koenig et al., 1997), female physical condition was assessed on a monthly basis on a seven-point scale (1 = meager, to 7 = fat) by E.L. and C.B. The assignment of females to different point categories was based on the degree of protrusion of skeletal elements such as the spinal column, ribs, hips, and shoulder blades. Hence, physical condition was only assessed when females were standing or walking quadrupedally, during which proper inspection of skeletal elements could be made. While subjective in nature, this method of assessment has been applied to studies of colobines (Koenig et al., 1997; Larney and Koenig, 2007; Ziegler et al., 2000) and macaques (Berman and Schwartz, 1988) in which links have

been shown between physical condition and the likelihood of conception, as well as the speed of infant development.

Statistics

In order to test whether the availability of *Vitex* leaves and fruits predicted levels of fP, we ran a general linear mixed model (GLMM) (alpha level: $p < 0.05$) with log-transformed monthly fP values as the dependent variable, reproductive condition as a fixed factor, female ID as a random factor, and the average log availability of young leaves, young fruits, and mature fruits as covariates in the model (SPSS, Version 16). The model included data from all months (N = 18) during which both hormonal and phenological data were available. We recognize that without detailed feeding data, we are unable to pinpoint the exact plant source of phytoprogestins. However, the present analysis allows us to determine whether monthly fP levels in this population are *consistent* with *Vitex* seasonality.

To evaluate the effect of elevated progestins on reproduction, we used data from Phase II of the study, when frequent hormone and behavioral data collection allowed us to determine receptive periods and ovarian cycling patterns. We identified the date at which fP began its seasonal elevation and divided the study period into (1) a "normal" progestin period (Jan 1 – April 15, 2006), and (2) a high progestin period (April 16 – September 28, 2006), as defined by patterns of fP in non-pregnant females (see results). We then conducted non-parametric tests (Mann Whitney U; 1-tailed, alpha level: $p < 0.05$; Sokal and Rohlf, 1995) to assess whether cycle length, the follicular phase of the cycle, the length of the receptive period, the length of the inter-mating period, or the probability of conception were significantly shorter during the normal progestin period compared to the high progestin period. Sample sizes were insufficient to statistically assess differences in the length of the luteal phase. For differences in cycle length and follicular phase, we categorized cycles and follicular phases that overlapped the high progestin period as belonging to that period. We did this because inhibition of ovulation should occur via increases in the follicular phase (e.g., Heistermann et al., 2004), either by eliminating or delaying ovulation. Since overall cycle lengths were calculated from one ovulation to the next, each cycle incorporated the luteal phase of the previous

ovulation, and the follicular phase of the current. There was however, one exception to this rule. Female B5 ovulated on April 18, 2006. Because this would only entail a two-day overlap with the high fP period, and because calculations of ovulation dates via fE patterns alone are prone to some degree of error, we decided to proceed on the side of caution and include this cycle and follicular phase as part of the normal fP period. Assuming that similar proximate mechanisms influence the duration of inter-mating intervals, we also categorized inter-mating intervals that overlapped the high progestin period as belonging to that period. For analysis of the probability of conception during each period, we categorized the April 18th B5 ovulation as belonging to the high progestin period. We did this because a positive effect of elevated progestins on conception is likely to occur shortly after ovulation (see Guyton and Hall, 2000), when implantation might occur. However, we ran the analysis with both categorization schemes to confirm results.

We used non-parametric tests (Mann Whitney U; 1-tailed, alpha level: $p < 0.05$; Sokal and Rohlf, 1995) to evaluate the impact of PC on reproduction. We included two values of PC in our analyses: (1) raw monthly ratings, and (2) ratios of PC ratings from the next month, divided by PC ratings from the present month. The second value was a calculation of the change in PC, or energetic flux. A value over 1 represents increasing PC, while a value under 1 represents decreasing PC. We then assessed whether female months when ovulations occurred were characterized by higher raw PC ratings or PC ratios compared to female months when ovulation did not occur. Female months were defined as all months from January 2006, up until the month of conception. We conducted the same comparison for female conceptive months per every ovulatory month.

RESULTS

Seasonal Pattern of Fecal Progestins

We found a distinct seasonal pattern in fP levels over the course of 20 months (February 2005 – September 2006), irrespective of reproductive condition. These increases occurred in similar periods in both years (April – September), although the exact timing differed from year to year. The hormone profiles for six non-pregnant

females that gave birth between late 2004 and early 2005 indicate a sharp rise in fP values between late May/early June and September, 2005 (Fig. 3.2). In 2006, two of these females (B2, B6) conceived in March and exhibited the expected fP and fE elevations associated with pregnancy. However, four additional females (Fig.3.2: B3, B5, B9, B12) exhibited hormone patterns consistent with another fP rise around a similar time period in 2006. Since fE levels remained at baseline, this elevation could not be explained by conception. Frequent samples from cycling females allowed us to pinpoint the timing of this 2006 rise to mid-April (April 16-19; Fig. 3.3a, 3.3b).

The hormone profiles for four females who conceived early in 2005 and gave birth between 2005 and early 2006 showed similar seasonal patterns (Fig. 3.4). Three of these females conceived between May and June 2005 (B4, B7, B10) and exhibited fP levels during the initial months of pregnancy that were comparable to, or higher than, the rest of pregnancy. The remaining female (B11) also showed an fP rise at the same time period, even though fE levels show that she had not conceived yet. In all four cases, fP levels stay elevated between May/June and September 2005, coinciding with the fP rise from the first set of females who were non-pregnant at this time (Fig. 3.2). Finally, in all four females who gave birth in 2005/2006 (Fig. 3.4), an fP rise clearly occurs again between Apr/May and September, 2006, even though baseline fE levels again indicate that none of these females had conceived.

Based on the patterns of fP levels in non-pregnant females, we therefore could identify one major period of elevated fP levels during each year (also see Fig. 2.1, chapter 2). Specifically, in 2005, we found a rise from baseline levels, around May/June and lasting through September. In 2006, a rise in fP levels began in April (16-19) and lasted again through September (the end of the study period) (Fig. 3.3a, 3.3b, see Fig. 2.1, chapter 2).

Are Patterns Consistent with *Vitex* Availability?

Phayre's leaf monkeys regularly fed on the young leaves and fruits of *Vitex* species during periods of elevated fP. In the absence of feeding data, we explored the possibility that seasonally elevated fP levels were due to phytosteroid consumption from *Vitex* species by evaluating whether *Vitex* leaf and fruit availability were linked to

monthly fP levels. Our results indicate that *Vitex* young leaf (GLMM, df = 1, F = 12.604, $p < 0.001$) and mature fruit (df = 1, F = 14.963, $p < 0.001$) availability, along with reproductive state (df = 1, F = 102.633, $p < 0.001$) explain a significant portion of the variation in monthly female fP levels (see Table 3.1 for full model summary). Indeed, a relationship between *Vitex* leaf and fruit availability and fP levels was clearly seen, even in non-pregnant (cycling or lactating) females (Fig. 3.5), suggesting that the rise in fP levels was independent of reproductive condition. Note however, that in both years, *Vitex* availability preceded the rise in hormone levels.

Reproductive Consequences of Fecal Progestins

The hypothesis that periods of seasonally elevated fP levels might have consequences for reproduction was evaluated using data from the 2006 season (Phase II). Specifically, we predicted that elevated fP would lead to increased cycle lengths and decreased sexual behavior, as measured by the duration of receptive and inter-mating periods, and increased probability of conception when ovulation occurred. We found that cycles overlapping the high progestin period (N = 7, mean = 50.6 ± 7.8 days, median = 49, range = 28-90; Mann Whitney U Test: U = 13.0, z = -2.313, p = 0.01) were longer than cycles that did not overlap this period (N = 11, mean = 31.2 ± 3.1 days, median = 28, range = 21-59; Fig. 3.6). This difference was mirrored by a longer follicular phase during the high progestin period (N = 7, mean = 37.9 ± 7.2 days, median = 35, range = 18-76) compared to the normal period (N = 11, mean = 18.0 ± 2.9 days, median = 15, range = 12-44; Mann Whitney U Test: U = 7.5, z = -2.813, p = 0.003), but no differences in the luteal phase (high progestin: N = 4, mean = 11.3 ± 1.7 days; median = 11.5, range = 7-15; normal: N = 14, mean = 12.5 ± 0.4 days, median = 12, range = 11-17). In terms of sexual behavior, we found no differences in the length of the receptive period (high progestin: N = 12, mean = 4.3 ± 0.9 days, median = 4.5, range = 1-12; normal: N = 23, mean = 4.2 ± 0.6 days, median = 4.0, range = 1-13; Mann Whitney U Test: U = 129.5, z = -0.301, p = 0.771). However, inter-mating intervals that overlapped the high progestin period (N = 11, mean = 22.2 ± 4.3 days, median = 15, range = 3-4) were significantly longer than those that were found during the normal progestin period (N = 17, mean =

11.8 ± 1.7, median = 10, range = 4-29; Mann Whitney U Test: U = 52.5, z = -1.931, p = 0.03, Fig. 3.7).

Despite the prolonged cycle lengths and inter-receptive periods, however, ovulations still occurred during the high progestin period. If we include the one ovulation that fell at the boundary between the two periods as belonging to the high progestin period, then nine of the 25 ovulations (36%) observed during the 2006 season occurred during the six months when fP levels were high. Of these nine ovulations, five were conceptive (55.6%). This compares to two conceptions out of 16 ovulations (12.5%) during the normal period. Therefore, the probability of conception was indeed greater when fP was high (Fisher's Exact Test, p = 0.03). If we re-categorized the April 18 ovulation as belonging to the normal progestin period, the relationship became even stronger (Fisher's Exact Test, p = 0.009).

The Effect of Physical Condition

Because food-related effects on female reproduction can also be more generally explained by changes in female energetic status (Ellison, 2003), we also evaluated whether reproductive patterns were consistent with monthly female physical condition, with decreasing or low physical condition associated with elongated cycle lengths, and increasing or good physical condition associated with a greater probability of conception. We found that this was clearly the case for conceptions, but not necessarily for ovulations. Specifically, although ovulatory conceptive months were not characterized by higher PC compared to ovulatory non-conceptive months (N = 7 conceptive months, N = 18 non-conceptive months; Mann Whitney U Test, U = 67.0, z = -.406, p = 0.444), ovulatory conceptive months were characterized by higher PC ratios (i.e. flux) (Mann Whitney U Test, U = 17.0, z = -2.881, p = 0.004). By contrast, no significant differences were found when assessing either PC or PC ratios in relation to months with and without ovulations (N = 25 ovulatory months; N = 7 non-ovulatory months; PC: Mann Whitney U Test, U = 57.5, z = 0.348, p = 0.745; changing PC: U = 81.5, z = -0.124, p = 0.908). This is despite the fact that four of five females who did not conceive by the beginning of the high progestin period in mid-April 2006, experienced extended cycle lengths between April and May, associated with declining or low PC (Fig. 3.8).

DISCUSSION

Consistent with previous reports on wild baboons (Higham et al., 2007) and chimpanzees (Thompson and Wrangham, 2008), our results indicate that female Phayre's leaf monkeys in Phu Khieo Wildlife Sanctuary experienced annual periods of seasonally elevated progestin levels, and these seasonal peaks coincided with periods of *Vitex* leaf and fruit availability. Although detailed feeding data were unavailable for the study period, these results parallel previous findings that *Vitex* consumption leads to elevated progestin levels in primates (Higham et al., 2007; Thompson et al., 2008) and humans (reviewed in Girman et al., 2002). As in the study on baboons (Higham et al., 2007), our results suggest that both *Vitex* leaf and fruit consumption influence endocrine function. Future studies comparing intake rates with chemical analyses of plant parts would be necessary to assess the exact *Vitex* species and plant parts affecting progestin levels.

Our results also supported the predicted effects of *Vitex* and elevated progestin levels on female reproduction. Specifically we found that cycle lengths, follicular phases, and inter-mating intervals that overlapped the high progestin period were longer than those that did not overlap the period, while luteal phases and overall lengths of the inter-mating interval were no different between the high and normal progestin periods. These results support previous findings that *Vitex* (Higham et al., 2007) and elevated progestin levels (synthetic or otherwise) (e.g., Bettinger et al., 1997; Steklis et al., 1982) impair primate ovarian and sexual function, and parallel previous studies showing that extended cycle lengths are due to the elongation of the follicular phase rather than the luteal phase (e.g., Czekala et al., 1988; Heistermann et al., 2004). The finding that the length of the receptive period was no different between the two time periods suggests that the frequency of receptive periods was perhaps a better measure of reproductive function. Nevertheless, although ovulations were less frequent during the high progestin period, they did not seem to be of lower quality in terms of fertility. In fact, following our expectations, we found that the period of elevated progestins was associated with a four-fold increase in the probability of conception when ovulation occurred. This finding supports clinical studies on humans (Milewicz et al., 1993; Westphal et al., 2004)

indicating that *Vitex* consumption can lead to increased progesterone levels and thus improved chances of conception.

In addition, however, when we analyzed the pattern of monthly physical condition, we found that patterns of reproduction, conceptions in particular, were also consistent with an energetic explanation (Ellison, 2003). Specifically, in months when ovulations occurred, conceptive months were characterized by higher ratios of changing PC compared to non-conceptive months. Although we did not find non-ovulatory months in general to be characterized by low or decreasing physical condition, four females exhibited periods of extended cycle lengths or anovulation around April/May 2006, associated with decreasing PC. Coincidently, this occurs at the same time period as the beginning of the high progestin period, suggesting perhaps that both factors might have influenced reproduction at this time.

Indeed, although it has often been suggested that phytosteroids play an important role in influencing the timing of reproduction in wild populations (Wallis, 1997; Whitten, 1983), Knott (2001) has argued that any relationship found between specific plant consumption and reproduction might simply be spurious, and due to the more general effects of overall food consumption on body condition. As other authors have noted, separating the effects of phytosteroids and nutritional condition on reproduction may prove to be extremely difficult in wild populations, where reproduction is tightly constrained by overall food availability (Knott, 2001; Thompson et al., 2008).

Proximate Mechanisms: *Vitex*, Progestins, and Reproduction

Despite this general difficulty, however, several studies focusing on both the chemical composition and the proximate effects of *Vitex* compounds have suggested that ingesting the chemical constituents from *Vitex* plants can indeed affect reproductive function. Studies on *V. agnus castus* (Iino et al., 2007; Sadenkrehula et al., 1991; Simons and Grinwich, 1989; Whitten, 1983) and *V. doniana* (Higham et al., 2007) have found progestin-like constituents in the fruits and leaves (*V. doniana*) of these species. Therefore, the measurement of fecal progestins might, in part, reflect the levels of the plant itself passed through the digestive system. However, based on other studies of

phytosteroid consumption (e.g., Rochester et al., 2009), it is expected that at least some level of absorption should occur.

Progestin-like compounds might directly interact with endogenous progesterone receptors, causing agonistic effects. As suggested by Higham et al (2007), the action of these compounds might resemble those of progestin-based contraceptives (reviewed in Landgren, 1990), down-regulating the production of lutenizing hormone, hence inhibiting ovulation. If this is indeed the case, it is likely that these phytoprogestins, like phytoestrogens (reviewed in Whitten and Naftolin, 1998), do not have the same affinity for progesterone receptors as endogenous progesterone itself. A lower binding affinity may explain why extremely high excreted progestin levels found in the present study did not completely inhibit ovulation. Because progesterone functions to prepare the uterus for implantation (Guyton and Hall, 2000), moderate levels of absorption, at which ovulation is not impaired, may then increase the chances of conception.

Experimental evidence on humans also shows that the ingestion of *V. agnus castus* fruit extract can increase endogenous progesterone levels by enhancing the ratio of lutenizing to follicle-stimulating hormone (Girman et al., 2002). Other studies show that that extracts of both *V. agnus castus* (Liu et al., 2001, 2004) and *V. rotundifolia* fruit (Hu et al., 2007) can bind to α- and ß-estrogen receptors and upregulate progesterone receptors. Furthermore, *V. agnus castus* also has well-documented inhibitory effects on prolactin (Milewicz et al., 1993; Sliutz et al., 1993; Wuttke et al., 2003), and clinical studies conclude that female fertility is improved because anti-prolactin effects lead to increased luteal phase progesterone levels (Milewicz et al., 1993). Therefore, even in controlled experimental conditions, where measurements of circulating hormones are available, the hormonal mechanisms underlying *Vitex*-related effects on reproduction prove to be complex.

Implications: *Vitex* and Phytosteroids in General

Our finding that seasonal increases in fP levels are related to *Vitex* availability is consistent with previous studies showing that plant parts of two other *Vitex* species (*V. doniana*: Higham et al. 2007; *V. fischeri*: Thompson et al. 2008) might have the potential to alter excreted progestin levels in wild primate populations. Furthermore, extracts from

at least three *Vitex* species (*V. agnus castus:* e.g., Liu et al., 2001, Sliutz et al., 1993; *V. negundo*: Bhargava, 1989, Das et al., 2004; *V. rotundifolia*: Hu et al. 2007) have been shown to alter either female or male reproductive hormone levels in captive animals. Thus, further experimental work on *Vitex* consumption is necessary to elucidate both the mechanisms leading to reproductive effects, and the species/plant parts that might induce these effects.

These experimental studies may be particularly relevant to our interpretation of reproduction in wild primate populations. *Vitex* contains hundreds of species (N = 250-467), distributed in both temperate and tropical regions such as Central/South America, Central Africa, and Southeast Asia (http://data.gbif.org/species/13194840), with the parts of many of these species forming important components of the diet of several primate populations (reviewed in Thompson et al., 2008). Furthermore, other plant genera commonly consumed by primates, such as *Acacia* (Garey, 1991; Garey et al., 1992), and *Pterocarpus* (Benie and Thieulant, 2003), have been shown to have phytosteroid-like properties. Although the influence of phytosteroids and those of energetic condition may be difficult to separate in the wild, it is crucial to assess the chemical composition and the effects of consuming such species to determine whether observed seasonality in primate reproduction is solely due to changes in nutritional status, or is due to the additive effects of both phytochemicals and nutritional condition.

Table 3.1 Results from General Linear Mixed Model (GLMM) for the influence of reproductive status, female ID, and *Vitex* spp. availability (young leaves, mature fruit, and young fruit) on log-transformed monthly female fecal progestin levels levels.

Factors	**df**	**F**	**p**
Intercept	1	189.65	< 0.001
Reproductive status	1	102.63	< 0.001
Female ID	9	0.54	0.845
Young leaves	1	12.60	< 0.001
Mature fruit	1	14.96	< 0.001
Young fruit	1	3.39	0.067

Figure 3.1. Rainfall at the study site between 2005 and 2006 (gray = monthly rainfall (mm), solid line = days with rainfall).

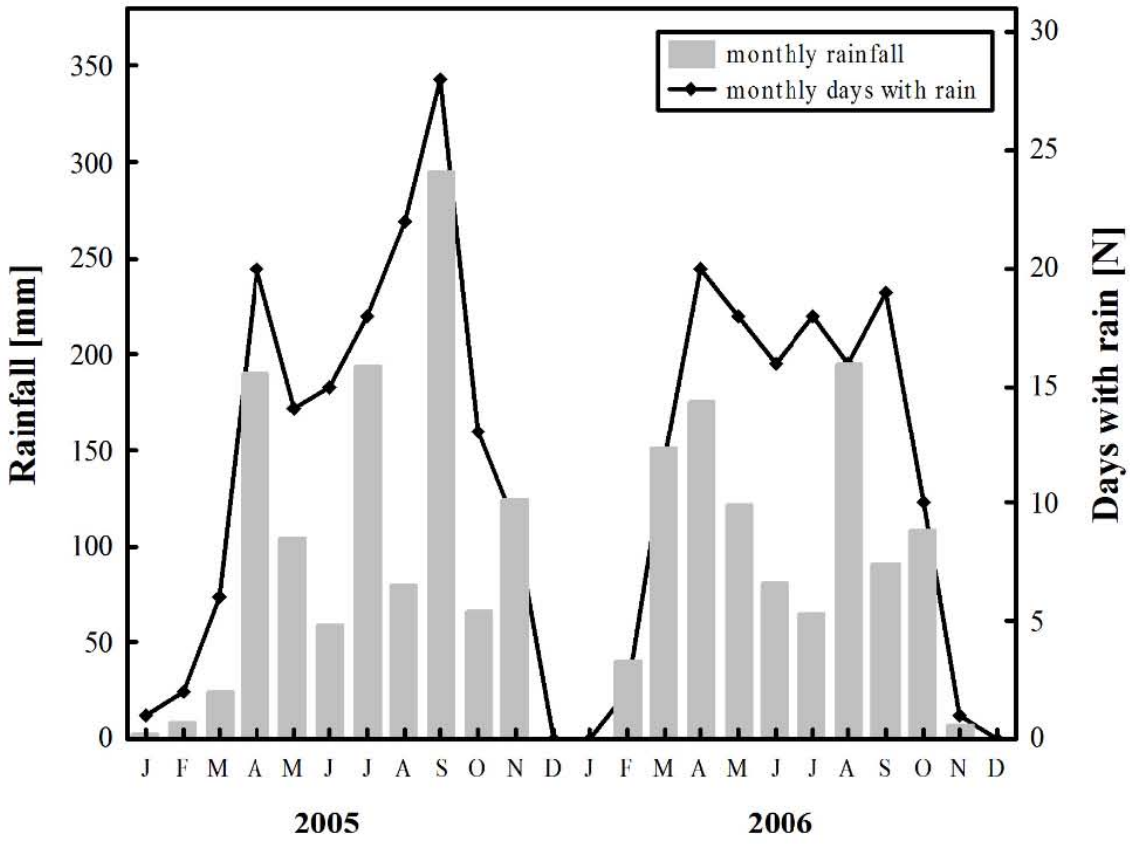


Figure 3.2. Reproductive hormone patterns for individual females (B2, B3, B5, B6, B9, B12) who gave birth between late 2004 and early 2005 and conceived again in 2006. Females arranged in two columns (left = last birth prior to the hormone collection period; right = last birth in the beginning of 2005; black bar = pregnancy; dotted line = fP (µg/g feces); solid line = fE (ng/g feces))

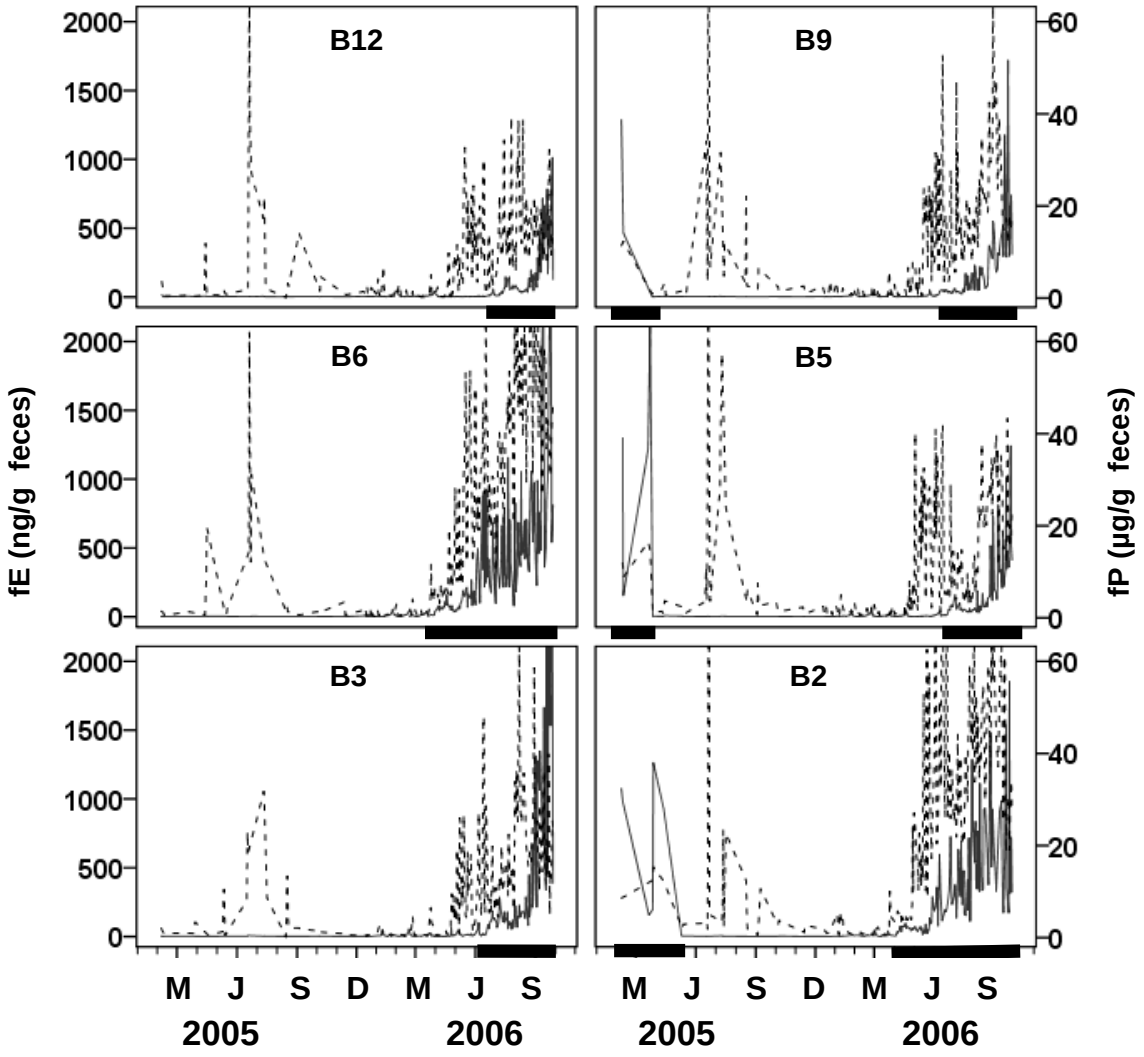


Figure 3.3a. Representative hormone patterns for female B3 who conceived in 2006 during the high progestin period (dotted arrow = beginning of high progestin period; black bar = pregnancy; dotted line = fP (µg/g feces); solid line = fE (ng/g feces)).

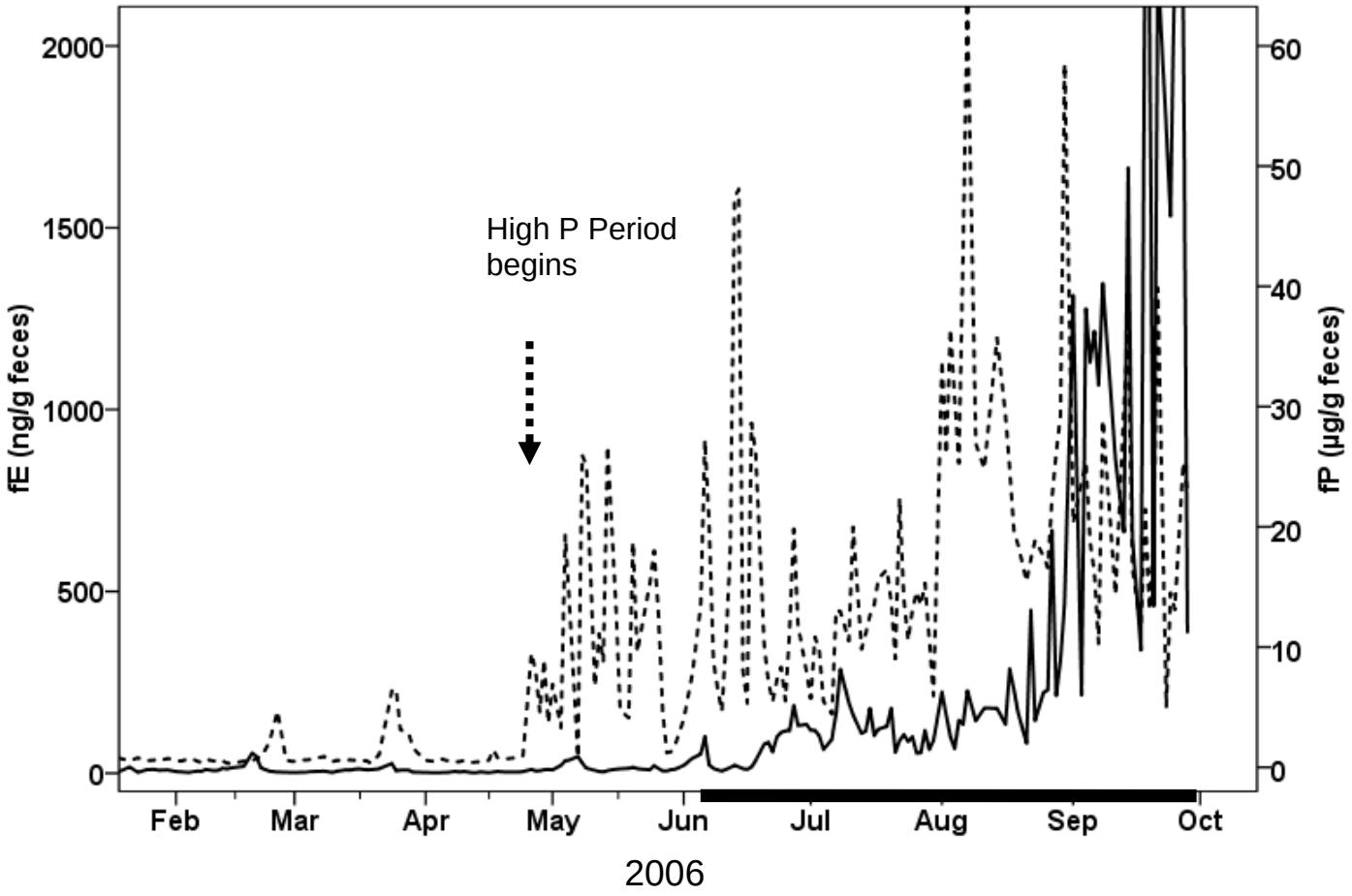


Figure 3.3b. Representative hormone patterns for female B5 who conceived in 2006 during the high progestin period (dotted arrow = beginning of high progestin period; black bar = pregnancy; dotted line = fP (µg/g feces); solid line = fE (ng/g feces)).

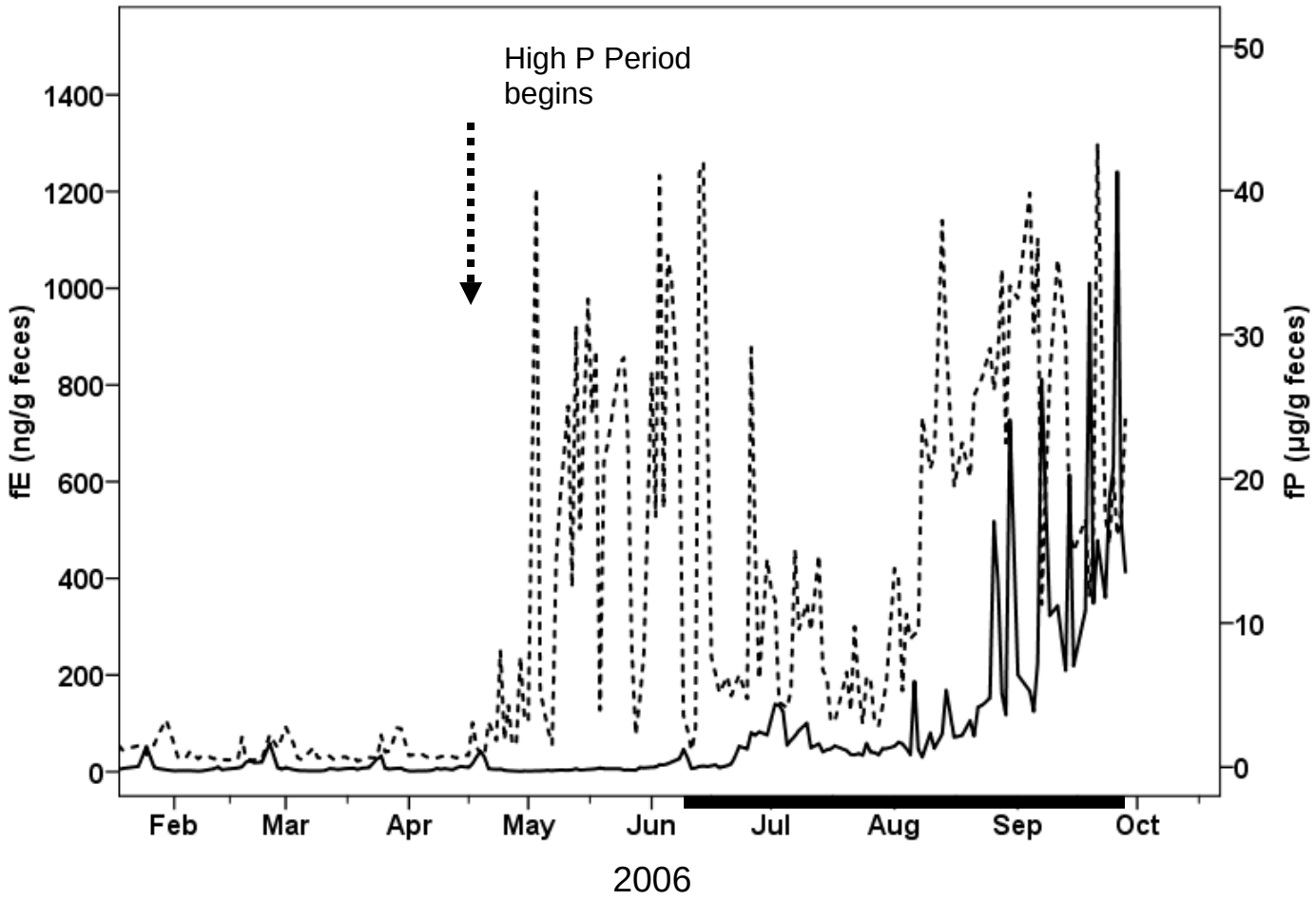


Figure 3.4. Reproductive hormone patterns for individual females (B4, B7, B10, B11) who conceived in 2005 and gave birth between late 2005 and early 2006 (black bar = pregnancy; dotted line = fP (µg/g feces); solid line = fE (ng/g feces)).

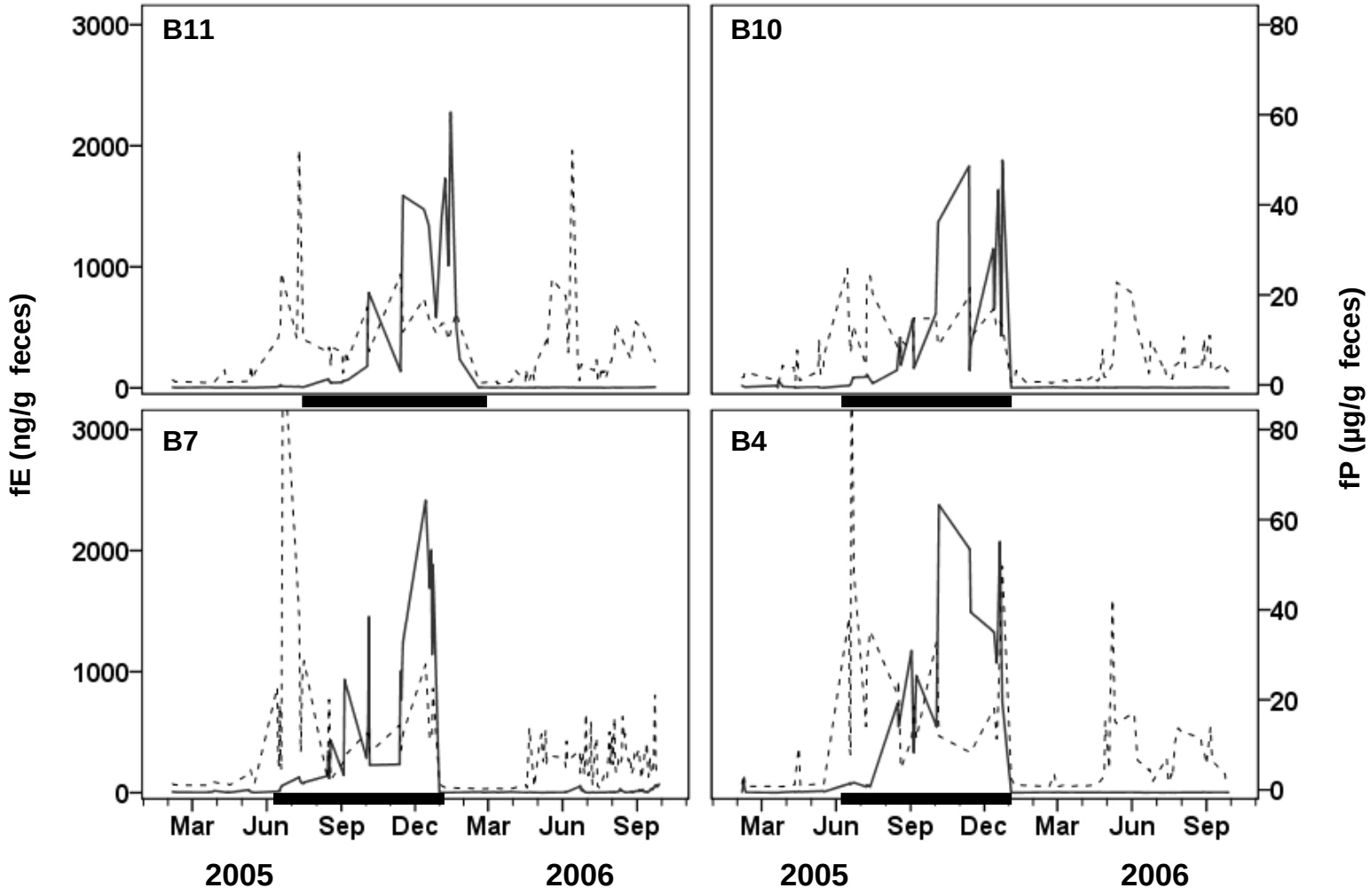


Figure 3.5. Average monthly fecal progestin levels of non-pregnant females in relation to monthly availability of *Vitex* spp. young leaves, mature fruit, and young fruit (bars = fP (µg/g feces), stacked lines = availability of *Vitex*, solid black line = young leaves, solid gray line = mature fruits, dotted black line = young fruits).

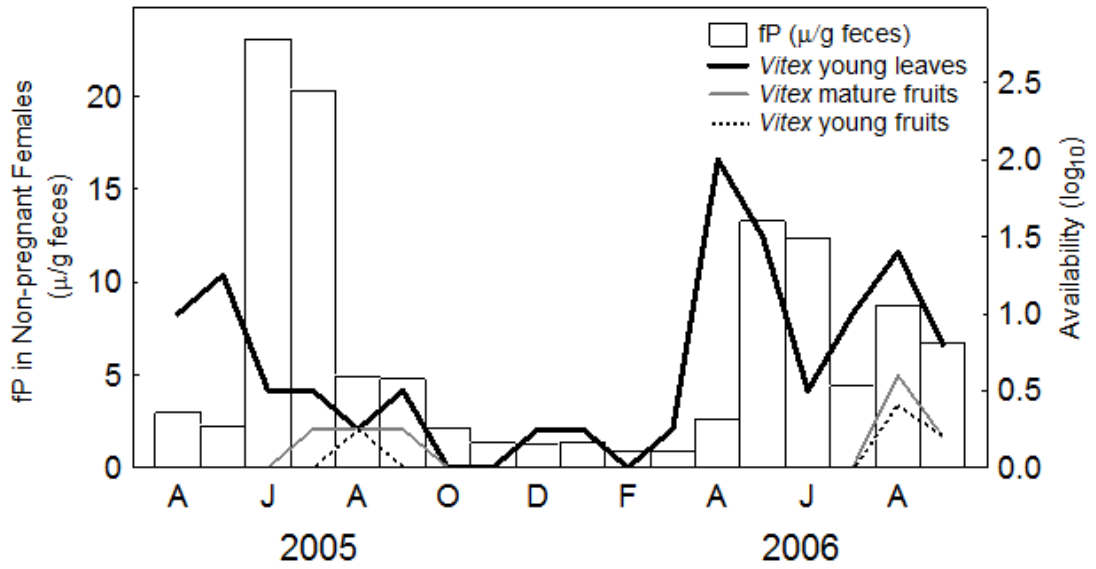


Figure 3.6. Cycle lengths during the normal progestin period compared to those that overlapped the high progestin period (solid line = median, box = interquartile range, error bars = 95% confidence intervals).

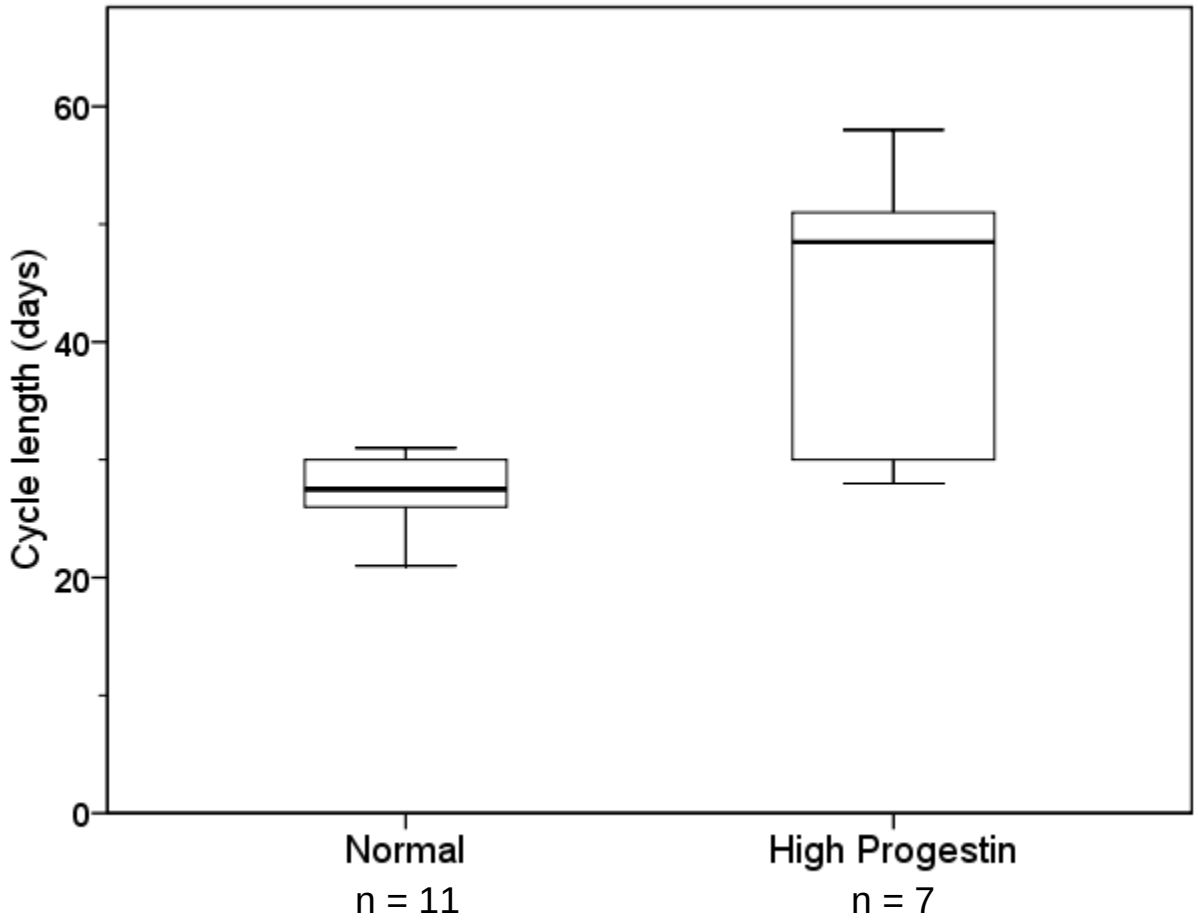


Figure 3.7. Length of the inter-mating interval during the normal progestin period compared to the high progestin period (solid line = median, box = interquartile range, error bars = 95% confidence intervals).

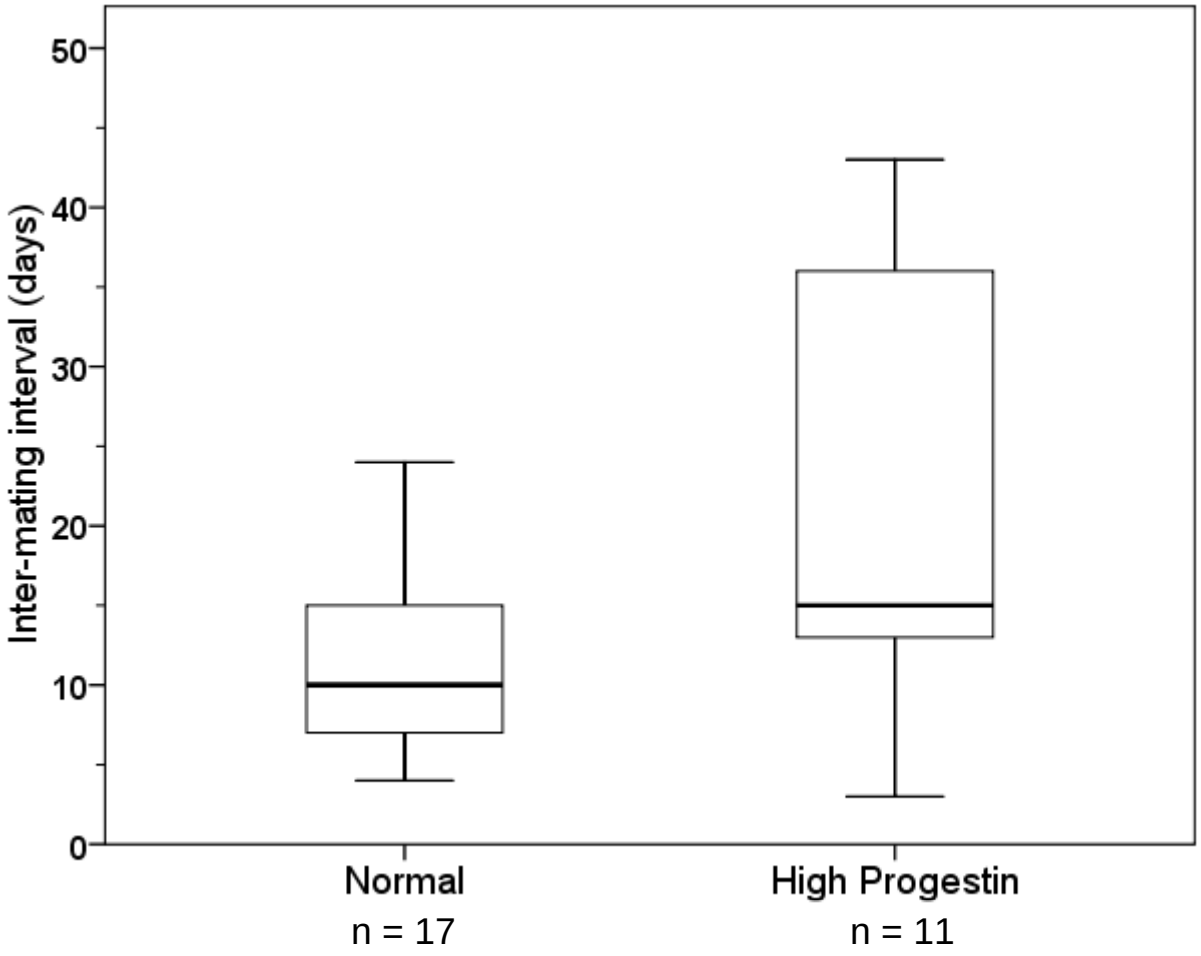
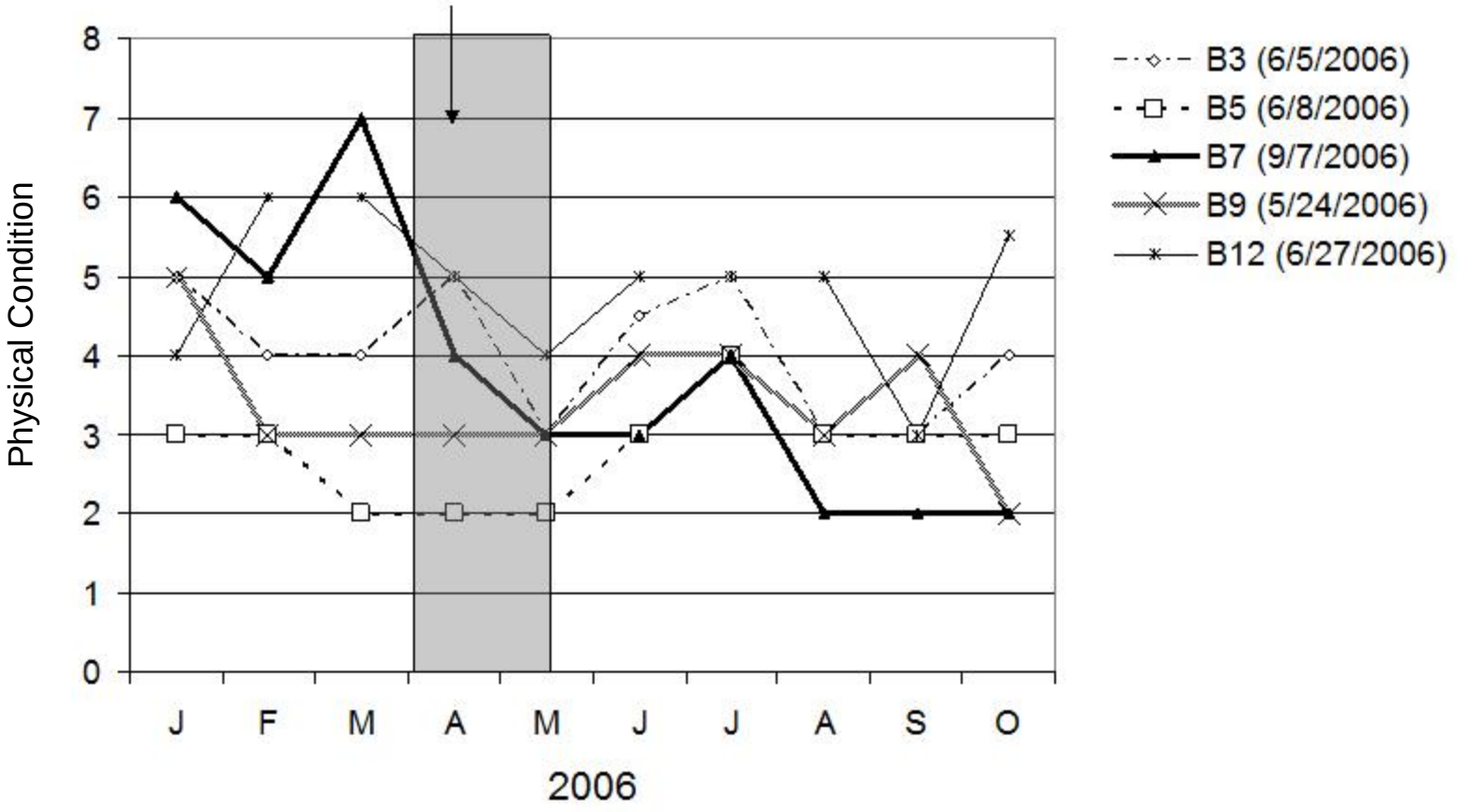


Figure 3.8. Monthly physical condition for five females who did not conceive by the beginning of the high progestin period in 2006 (individual lines = different females (conception date listed in legend for each female); arrow = beginning of high progestin period; solid gray = period of time when four of five females had anovulatory periods).



CHAPTER FOUR

PATTERNS OF NON-FERTILE MATING IN FEMALE PHAYRE'S LEAF MONKEYS

Abstract

Primate females are known to be sexually receptive outside of the periovulatory period and even during gestation. This continuous receptivity, especially in relation to multi-male mating, has been hypothesized to increase paternity confusion, and thus lower the risk of infanticide, or increase the chances that some males will provide other benefits such as infant care (van Noordwijk and van Schaik, 2000). However, many studies suggest that females often provide some honest information about the timing of ovulation. It has been suggested that this pattern of honesty coupled with extended receptivity might be due to the balance of two different mating strategies: (1) mating with high quality males during peak fertility on the one hand, with (2) paternity confusion directed toward other males during periods of lower fertility on the other hand (Stumpf and Boesch, 2005; van Schaik et al., 2000). Futhermore, although non-fertile receptive periods are found in many primates, the prevalence of receptivity during certain temporal periods (e.g., follicular phase) relative to others suggests that sexual behavior is still to some degree under hormonal control. Here we investigate patterns of mating and female mate choice in a wild group of Phayre's leaf monkeys (*Trachypithecus phayrei crepusculus*) (Phu Khieo Wildlife Santuary, Thailand) containing one adult and one subadult breeding male. We specificially assessed whether receptivity was more common during certain reproductive periods relative to others, whether females conveyed any honest information about the timing of fertility, and whether the timing of receptivity (fertile or non-fertile) influenced female mate preferences. Over an eight month study period, we collected data on sexual behavior from seven adult females via focal and *ad libitum* sampling. We then analyzed these behavioral data in relation to the timing of ovulation and the periovulatory period based on fecal hormone analysis. We found that

female receptivity was more frequent around ovulation compared to the rest of the cycle, but that non-fertile receptivity peaked during specific time periods (e.g., follicular and perimenstrual periods, days 50-100 of gestation), suggesting that receptivity was not independent of hormones. We also found that while characteristics of receptive periods such as receptive period length and the length of the inter-mating interval preceding each period were not signifantly different between non-fertile cycling receptive periods, fertile receptive periods, and post-conceptive receptive periods, fertile cycling periods were characterized by the highest rates of proceptive and receptive behavior. Finally, consistent with the hypothesis that females utilized a mixed mating strategy, we found that proceptive and receptive behavior more frequently involved the adult male during fertile periods and periovulatory days, but the subadult male during post-conceptive periods. However, the same preference for the subadult male was not found for non-fertile receptive periods. Since most conceptions occurred during the latter half of the study, one possible explanation for this pattern is that females employed a strategy more biased towards honesty when the subadult male was younger, but switched to a strategy more biased towards paternity confusion as he matured. Because females of this species are characterized by a high lactation to gestation ratio but infanticide has never been documented, we propose that non-fertile mating in Phayre's leaf monkeys may be a strategy used successfully by females to confuse paternity and thus decrease the probability that males will commit infanticide. In addition, females might offer mating opportunities in return for male services such as "infant care." Evaluation of the latter hypothesis must await future studies focusing on mating patterns in relation to male-infant relationships.

INTRODUCTION

Mammalian sexual behavior has been traditionally divided into three components: (1) proceptivity, (2) receptivity, and (3) attractivity (Beach, 1976). Proceptivity is a measure of female sexual motivation and/or initiation, and has most often been quantified by the frequency of female sexual solicitation (e.g., presenting of the hindquarters in mammals: Beach, 1968; "lipsmacking" in baboons: Saayman, 1970; "headshuddering" in Hanuman langurs: Sommer et al., 1992). Receptivity is a measure of the degree to which females physically facilitate copulation, and has often been quantified by the frequency of mounting and copulatory behavior. Finally, attractivity considers the female's stimulus value to the male. Attractivity is strongly influenced by visual (Barelli et al., 2007; 2008), olfactory (Cerda-Molina et al., 2006), and behavioral (Engelhardt et al., 2005) signals emitted by the female, and is often measured by the degree to which males show interest in females (e.g., approaches, genital inspections).

Among most mammals, all three aspects of female sexual behavior are generally constrained to the period of "estrous" (Heape, 1900), when gonadal hormones simultaneously stimulate ovulation and sexual behavior (Wallen, 2001). Under the influence of ovarian hormones, females become more proceptive and receptive, and males, consequently, also become more attracted to females. Indeed in many mammalian species (e.g., rats, sheep), receptivity is physically impossible without the periovulatory surge in estradiol (Wallen, 2001). This tight coupling of behavior and reproductive function ensures that copulation occurs when conception is likely.

While patterns of female sexual behavior in strepsirrhine primates follow the general mammalian pattern (e.g., Eaton et al., 1973; Shideler et al., 1983), sexual behavior in haplorrhine primates is less temporally restricted. Abundant studies show that female haplorrhines can be sexually receptivity throughout the ovarian cycle (Barelli et al., 2008; Brauch et al., 2007; Carnegie et al., 2004; Sommer et al., 1992; van Belle et al., 2009), often having receptive periods that are much longer (e.g., *Pan troglodytes*: 7-17 days: Tutin 1975; *Papio anubis*: 15-20 days: Smuts, 1985; *Semnopithecus entellus*: 7-9 days: Borries et al., 2001; see summary in van Schaik et al., 1999) than the few days around ovulation when conception is most likely. In species where females have sexual swellings, the period of tumescence often also extends beyond the fertile period (Barelli

et al., 2007; Deschner et al., 2004). Finally, in the majority of primate species, females also copulate (Carnegie et al., 2005; Engelhardt et al., 2007; reviewed in van Noordwijk and van Schaik, 2000; van Schaik et al., 1999) or have tumescent swellings during pregnancy (Barelli et al., 2008; Wallis and Lemmon, 1986), when conception is impossible. This constant capacity for receptivity and advertisement for mating has led scientists to categorize haplorrhines in general and humans in particular, as species with "concealed ovulation" (Pawlowski, 1999; van Schaik et al., 2000; see Sillen-Tullberg and Møller, 1993 for alternative definition).

There is some evidence to suggest, however, that non-fertile receptivity, whether during cycling or pregnancy, is not completely independent of the proximate influence of hormones. For instance, non-fertile receptivity is often found to be more common during the follicular phase of the ovarian cycle (e.g., O'Neill et al., 2004; reviewed in Dixson, 1998), when estrogen levels are increasing, follicular maturation occurs, and there is some probability, albeit low, of conception. In humans (Ford and Beach, 1951; Udry and Morris, 1968) and non-human primates (Borries et al., 2001; Loy, 1970; Sommer et al., 1992), studies have also found a peak in sexual behavior prior to, or at the onset of menstruation. Loy (1970) has suggested that this perimenstrual mating peak might be due to a secondary rise in estradiol during the late luteal phase, as documented in humans (O'Connor et al., 1974) and some catarrhines (e.g., Shimizu et al., 2003). Similarly, within most species of haplorrhines, post-conceptive sexual behavior is often constrained to a particular timeframe (*Cercocebus torquatus*: Gordon et al., 1991; *Semnopithecus entellus*: Borries et al., 2001; Sommer et al., 1992; *Trachypithecus cristatus*: Shelmidine et al., 2009), and this temporal restriction has often been explained as the result of rising estrogen levels (Gordon et al., 1991; Yan and Jiang, 2006) and/or high estrogen to progesterone levels (Bielert et al., 1976; Engelhardt et al., 2007). However, many studies have not found a direct correlation between hormone levels and receptivity, both in cycling (e.g., Brauch et al., 2007) and in pregnant females (e.g., Yan and Jiang, 2006), and hormones have failed to explain why sexual behavior is uncommon in the late stages of gestation, even though estrogen levels continue to rise (e.g., Bielert et al., 1976; Engelhardt et al., 2007).

On an ultimate level, several adaptive hypotheses have been proposed to explain continuous receptivity (reviewed in Pawlowski, 1999; Small, 1988; van Noordwijk and van Schaik, 2000), particularly in relation to multi-male mating (Hrdy and Whitten, 1987). These hypotheses posit that the emancipation of sexual behavior from the periovulatory phase of the cycle allows females to use behavior (or sexual swellings) as honest or dishonest indicators of fertility. Based on the general notion that communication should be selfish, selection should favor females (signalers) who use signals to manipulate the behavior of males (receivers), so long as the signal is not too costly to produce, and the result provides some benefit to the female (Dawkins and Krebs, 1978; reviewed in Gouzoules and Gouzoules 2002). Therefore, deceptive signals should be common, and at any given time, sexual behavior may or may not be an accurate indicator of the physiological state of the female.

The most prominent of these hypotheses focuses on the use of sexual behavior to conceal ovulation and thereby confuse paternity (Hrdy, 1979). This hypothesis proposes that by extending the period of receptivity, a female reduces the likelihood that a single male can monopolize matings, thereby reducing the certainty that any one male is the sire of her offspring. Paternity confusion is thought to function mainly to reduce the likelihood that males will commit infanticide (Hrdy, 1979). Primates, particularly cercopithecoids, seem to be highly vulnerable to infanticide because of their long lactation to gestation ratio. In species characterized by such high ratios, females do not exhibit post-partum estrus, and males benefit from committing infanticide because the killing of dependent infants brings lactating, amenorrhetic females back into estrus (van Noordwijk and van Schaik, 2000; van Schaik, 2000b). In addition, paternity confusion might also function to increase the probability that males will provide additional "services" to the female, including some degree of infant care. For instance, males might carry infants (Deag, 1980), tolerate infants at feeding sites (Hill, 1986), aid infants in social conflicts (Smuts, 1985), and even defend them against predators (Whitten, 1987) and infanticidal males (Borries et al., 1999; Crockett and Sekulic, 1984). In fact, extended receptivity and paternity confusion for "material benefits" has been hypothesized to explain patterns of non-fertile mating in primates, as well as other vertebrate species (e.g., birds: Rodriguez-Girones and Enquist, 2001).

Despite the multitude of ways that paternity confusion might benefit females, in many cases, studies have found that while primate females have the capacity for receptivity outside of the fertile period, the frequency of receptive behavior is actually higher around ovulation (e.g., Engelhardt et al., 2005; O'Neill et al., 2004; Ostner et al., 2006; van Belle et al., 2009, reviewed in van Schaik et al., 2000). Thus, in many species, female fertility is not completely concealed. Why would this be the case?

Female sexual strategies are hypothesized to be the result of two contradictory "choices" (see Stumpf and Boesch, 2005). On the one hand, it may be advantageous to (1) exhibit continuous receptivity, thereby confusing paternity as a counter-strategy to infanticide (Hrdy, 1979) and/or as a means to secure other benefits (reviewed in van Schaik and Paul, 1996). At the same time however, females may want to (2) honestly advertise fertility to ensure that mating with genetically superior males (Anderson, 1994; Clutton-Brock and McAuliffe, 2009; Maynard Smith, 1991) takes place when the likelihood of conception is high (Stumpf and Boesch, 2005). Given this mixed strategy (van Schaik et al., 2000; see Nunn 1999 for similar discussion on sexual swellings), females might be expected to prefer high-ranking males around ovulation, but show no preference or preference for lower-ranking males during non-fertile periods. Results from several studies have supported this hypothesis, with females preferentially proceptive and receptive to dominant males during the periovulatory period, while showing no preference, or favoring lower-ranking males (adult or subadult) outside of the periovulatory period (e.g., Barelli et al., 2008; Gesquiere et al., 2007; Stumpf and Boesch, 2005), and during pregnancy (e.g., Carnegie et al., 2004; Carnegie et al., 2005; Gordon et al., 1991). Indeed, preferences for traits such as dominance and masculinity during the fertile phase have even been found in studies on humans (reviewed in Gangestad et al., 2007; Gangestad and Thornhill, 2008).

In recent years, a plethora of studies have focused on female mating patterns and mate choice in wild primate populations (Barelli et al., 2008; Brauch et al., 2007; Carnegie et al., 2004; 2005; Deschner et al., 2004; Engelhardt et al., 2007; Engelhardt et al., 2005; Higham et al., 2008a; Higham et al., 2009; van Belle et al., 2009). Despite this interest, only two studies have examined patterns of extended receptivity relative to hormonally defined fertile periods in wild colobines (Harris and Monfort, 2006;

Heistermann et al., 2001b). Both of these studies focused on species in which infanticide is common (Borries, 1997; Hrdy, 1977; Oates, 1977).

In the current study, we use eight months of behavioral and hormonal data from seven females to investigate mating patterns and female mate preferences in a wild group of Phayre's leaf monkeys (*Trachypithecus phayrei crepusculus*) containing two sexually active males: one adult, and one subadult. Like all haplorrhines, female Phayre's leaf monkeys are characterized by a long lactation relative to gestation, suggesting that the risk of infanticide should be high (van Noordwijk and van Schaik, 2000). However, eight years of observations in the wild have yielded no reports of infant deaths due to male aggression (Koenig and Borries, unpublished data). These patterns suggest that females might employ successful counterstrategies to infanticide, including perhaps, the use of extended receptivity for paternity confusion.

Dispersal in this population is female-biased. Although males may also disperse, they more often (relative to females) mature and breed in their natal groups (Borries and Koenig, unpublished data). Multi-male groups contain only two to five adult males, with males forming clear dominance relationships. Young males are known to achieve alpha rank upon maturity, either due to rank change, or because the previous alpha male emigrated (Borries and Koenig, unpublished data). Hence, even a subadult male may pose a substantial threat to females and their dependent infants.

This study has three major aims: (1) First, we investigate the pattern of receptivity in female Phayre's leaf monkeys and determine if receptivity is temporally constrained, and thus related to proximate changes in hormones. Based on studies of other haplorrhines, we predict that non-fertile cycling and post-conceptive receptive periods will occur predominantly during constrained time periods (e.g., periovulatory period, follicular phase, perimenstrually, days 50-100 of gestation), perhaps related to hormonal changes. (2) Secondly, we aim to assess similarities or differences in non-fertile and fertile receptive periods and determine if ovulation is truly "concealed" in this species. We predict that if ovulation is completely concealed, characteristics of different categories of receptive periods, such as receptive period length, the length of the inter-mating interval prior to receptivity, and/or overall rates of proceptive and receptive behaviors might be similar, thus facilitating paternity confusion. Alternatively, any or all

traits can be dissimilar, consistent with a female strategy that provides some honest indication of fertility. (3) Finally, we evaluate whether females have preferences for different males during fertile, as opposed to non-fertile periods of receptivity. We predict that females will prefer the dominant adult male during fertile periods, but will show either no preference or preference for the subadult male during non-fertile periods.

METHODS

Study Site

Research was conducted in Phu Khieo Wildlife Sanctuary, in Northeast Thailand (16°5′-35′N, 101°20′-55′E; Chaiyahphum Province, elevation: 300-1300 m above sea level). The sanctuary encompasses an area of 1573 km^2, and is home to several groups of vertebrates, including primates (N = 7 species), felids (N = 8 species), viverrids (N = 10 species), and raptors (Borries et al., 2002; Grassman et al., 2005). The study site of Huai Mai Sot Yai is located 600-800 m above sea level at 16°27′N, 101°38′E. The site is composed of evergreen and dry dipterocarp forest (Borries et al., 2002), with annual rainfall averaging 1400 mm (Kumsuk et al., 1999).

Subjects & Study Period

Research took place within an ongoing study conducted by Andreas Koenig and Carola Borries (October, 2000 – present). Data in the present paper spans the study period from February 1 to September 28, 2006 and focuses on one group called PB. This group was fully habituated in spring of 2004, and demographic data have been collected since June, 2003 (Borries & Koenig, unpublished data). In the beginning of the study period, the group contained one adult male, one subadult male (with testes descended), 10 adult females, and 15 immatures. One additional male became subadult (testes descended) in April 2006. However, this later-maturing male was only observed to copulate twice (0.5% of total copulation events) during the entire eight-month observation period. Therefore, when comparing female preference, we only considered the adult male and the more mature subadult, who together accounted for 93.9% of all proceptive behaviors and 97.5% of all receptive behaviors, including 99.5% of all copulations.

Of the ten adult females in the study group, three were lactating for the majority of the study (two since the very beginning, and one as of February 3, 2006). Hormonal and behavioral data showed no evidence that these females returned to cycling within the study period. Of the remaining seven females, six began cycling prior to the beginning of the study. One additional female was lactating at the beginning of the study but resumed ovulation (based on behavioral and hormonal data) in June 2006. All seven females also conceived during the study period. Hence, our data on sexual behavior within cycling and pregnant females are derived from data collected only from these seven females (Table 4.1).

Fecal Sample Collection

In order to assess reproductive state and ovulation in females, a total of 1433 samples were collected during the study period. Of these samples, we used 1277 (1138 female days) collected from the seven study females to characterize ovulation, fertile phases, and conceptions (Table 4.1). These females were targeted at least every other day for sample collection, resulting in 22.3 samples/female/month for the duration of the study. Samples were collected in 30ml plastic vials (Sarstedt: vial #75.1337.500, lid #76.1340.560), kept on ice in the field, and frozen (-20ºC) upon return to the field station (within 2-13 hrs). In December 2006, samples were shipped on ice to the Conservation and Research of Endangered Species (CRES) at the Zoological Society of San Diego.

Fecal Hormone Analysis

At CRES, samples were lyophilized and sifted through mesh wire (16 x 16 mesh; McMaster-Car: #9223T82) to remove vegetative matter. Samples were then shipped to the Core Assay Facility at the University of Michigan for further extraction and radioimmunoassay. Samples were doubly extracted following protocols established by Wasser et al. (1994). Following extraction, samples were stored in methanol and frozen at -20°C until assay. Progesterone and estrogen metabolites were analyzed using RIA (chapter 2 for details). For both hormones, samples yielded dose-response curves parallel to the standard curve. Accuracy tests yielded recoveries of 104.1 ± 4.4% (N = 8) for progestins and 90.2 ± 10.0% (N = 6) for estrogens. Assay sensitivity was 31.25 pg/tube

(6.25 ng/ml) for progestins and 0.125 pg/tube (5 pg/ml) for estrogen, calculated as the lower limit at which serial dilutions of the lowest standard no longer yield changes in hormone concentration. For progesterone, intra-assay and inter-assay CVs for high (20% binding) and low (85% binding) fecal pools were 2.8% and 7.2%, and 9.9% and 16.3%, respectively. For estrogen, intra- and inter-assay CVs for high (20%) and low (80%) binding were 6.2% and 1.7%, and 6.0% and 10.6%.

Cycle Characteristics

Because fP levels were subject to seasonal plant-induced fluctuations that obscured cycling patterns for a portion of the study period (chapters 2 & 3), we used daily means of fE levels to construct hormone profiles for the seven females who cycled and conceived. We identified 20 fE peaks during this period, 19 of which were characterized by adequate sampling (every other day) around the day of the peak. For the one event occurring during a three-day sampling gap, we extrapolated the day of the peak based on trends in the rise and fall of fE levels prior to and following the gap (see chapter 2). In eleven cycles, we were able to identify a one-day (range: 0-2 days) difference between the fE peak and the postovulatory fP rise. We therefore assumed ovulation occurred shortly (within a day) after the ovarian surge in estrogen. Factoring a lag-time of 24 hours for circulating hormones to be metabolized and excreted in feces (Lu and Czekala, unpublished), ovulation likely occurred sometime between days -1 to 0 of the fE peak. We defined Day -1 as the ovulation date. Our definition of ovulation assumes that normal follicular fE patterns and fE peaks in particular were sufficient to initiate the surge in luteinizing hormone necessary for the release of the egg from the follicle (see chapters 3, 4).

Hererin we refer to the day of the fE peak as Day 0. Thus, one day before the fE peak is Day -1, one day after the peak is Day 1, and so forth. All days were assigned post-hoc once hormone data were available. The luteal phase was defined as the time period from Day 1 (the estimated rise in fP, see above) to the day prior to the next fE rise from its postovulatory nadir (chapter 2; Emery and Whitten, 2003). We assumed that the rise in fE from its nadir (2 SD above preceding three values) indicates the beginning of follicular development. Similarly, the follicular phase was calculated as the first day of the fE rise,

to the day of the next fE peak (Day 0). Conceptions were defined as ovulations that were followed by a continuous rise in fE levels (see chapter 2).

Human studies have found that ova rarely survive beyond 24 hours (France, 1981) and that most conceptions result from sperm that have been in the reproductive tract for no more than three days (Wilcox et al., 1995). Hence, many studies have defined the periovulatory period as five days long (e.g., Deschner et al., 2003; Engelhardt et al., 2005; Higham et al., 2008a), encompassing the three days prior to ovulation, the day of ovulation, and the day following ovulation, although more conservative estimates are also common (e.g., seven days: Carnegie et al., 2004; Harris and Monfort, 2006; van Belle et al., 2009). We defined the periovulatory period as a six-day window comprising the three days prior to ovulation, the day of ovulation, and the two days following ovulation (Fig. 4.1). We included two rather than one day following ovulation as part of the fertile period because ovulation likely occurred between days -1 and 0 relative to the fE peak, rather than day -1 per se.

Behavioral Data Collection

The study group was followed for 220 of the total 240 days of the study period, totaling 2603 contact hours. During group contacts, various observers collected focal and *ad libitum* data on sexual behavior (Altmann, 1974). Focal follows rotated through all adult females in a pre-determined order, changing subjects every 20 minutes. During focal follows, sexual behavior was recorded on a continuous basis. *Ad libitum* data were collected during focal follows and during periods when specific cycling females were targeted for the collection of fecal samples. Because the position of the observer changed throughout the day due to focal follows and the collection of fecal samples from specific females, we minimized the potential bias that individual females were sampled more than others in *ad libitum* collection of behavior data (see Borries et al., 1991).

Sexual behavior predominantly occurred in the early morning and late afternoon. If a female was observed to begin copulating (either when collecting focal or *ad libitum* data), we additionally made every effort to follow her during subsequent days until sexual behavior ceased.

We recorded attractive, proceptive, and receptive behavior (Beach, 1976); however, only proceptivity and receptivity were analyzed for the present study. Proceptive behavior included female presentation of the hindquarters, head shaking, and inviting (simultaneous presenting and headshaking). Receptive behavior included copulations (mount with intromission), as well as attempted copulations (mounting only) (Sommer et al., 1992). We recorded 406 copulations from the seven females included in the study. Copulation calls were never observed.

Analysis of Sexual Behavior in Relation to the Fertile Phase

Analysis of primate sexual behavior in relation to fertility has been traditionally conducted in two ways: (1) by defining behavioral receptive periods (Borries et al., 2001; Sommer et al., 1992) and assessing their temporal overlap with hormonally-defined periovulatory periods (Heistermann et al., 2001b; Pazol, 2003), and (2) by comparing daily sexual behavior rates outside of, and within hormonally defined fertile periods (Barelli et al., 2008; Brauch et al., 2007; Engelhardt et al., 2005). In order to facilitate comparisons with studies on other wild colobines (Borries et al., 2001; Sommer et al., 1992), particularly for the length of the receptive period, we chose the former method.

Following previous studies (Borries et al., 2001; Shelmidine et al., 2009; Sommer et al., 1992), receptive periods were defined as a set of consecutive days (not interrupted by more than two days) during which a female copulated, or attempted to copulate. A receptive period could be one day long only when at least one copulation was observed. The inter-mating interval was then calculated as the number of inclusive days from the first day following one receptive period, to the last day before the next receptive period. We distinguished three types of receptive periods (1) fertile, (2) non-fertile cycling, and (3) post-conceptive. Fertile receptive periods were those periods that overlapped the hormonally defined six-day period around ovulation. Non-fertile cycling receptive periods were defined as receptive periods occurring within any other portion of the ovarian cycle. Finally post-conceptive receptive periods were defined as receptive periods following the last fertile receptive period associated with conception.

Following Ostner et al. (2006), we used all sexual behaviors collected *ad libitum* and during focal follows to calculate rates of proceptive and receptive behavior during all

receptive periods. Although our data collection method minimized the potential bias that *ad libitum* sampling more commonly involved specific females relative to others, rates calculated from a combination of focal and *ad libitum* data collection should be interpreted with caution. Since 93.8% (137 of 146) of receptive period days were characterized by 10 or more observation hours (range 10.00-13.25 hours), and only one day (0.7%) was characterized by less than six hours of observation, we calculated daily, rather than hourly rates of behavior. We also assessed a simple measure of "involvement" in receptive periods for each male. A male was defined to be involved in a receptive period if he was engaged in any proceptive or receptive behavior with the female during that period.

Statistics

Because the number of receptive periods per female varied and was low compared to the number of comparisons made (effect of receptive period type and male identity), we treated each receptive period rather than each female as an independent data point. Using repeated measures of the same female introduces error due to pseudoreplication. Accordingly, results should be interpreted with caution. Because data were not normally distributed, all analyses were non-parametric (Freeman-Halton Extension of Fisher's Exact Test (2x3 table): Freeman and Halton, 1951; Kruskal-Wallis, Mann Whitney, Wilcoxon Signed Ranks, Pearson's Correlation: Siegel and Castellan, 1988) with alpha level set at $p = 0.05$. We used the Freeman-Halton Extension of Fisher's Exact Test (Freeman and Halton, 1951) to evaluate whether there was an association between the type of receptive period and whether or not males were engaged in any sexual behavior ("involved") with the female during that period. The Freeman-Halton Test is a generalized version of the Fisher's Eact test and is used to evaluate association in an RxC table with small sample sizes. The test is inherently two-tailed, with a low p value leading to the conclusion that the categories are significantly associated. Sequential Bonferroni Holm adjustments (Holm, 1979; Rice, 1989) were made to reduce the acceptable p value for multiple comparisons. We note the adjusted α value in the results when relevant. Similar adjustments were applied to all post-hoc comparisons. We performed two sets of post-hoc comparisons: (1) those that focused specifically on differences between fertile

receptive periods and each of the two other categories of receptive periods, and (2) those that looked within extended fertile receptive periods to investigate potential differences between periovulatory and non-periovulatory days. All analyses were performed in SPSS 16.0 (2007, SPSS Inc., Chicago, IL) or via a web-based program provided by Vassar College (VassarStats: http://faculty.vassar.edu/lowry/VassarStats.htm).

RESULTS

Temporal Distribution of Receptive Periods

We identified a total of 59 receptive periods, totaling 252 female days. These included 35 cycling receptive periods (N = 147 female days, 58.3% of all receptive periods; Table 4.2) and 24 post-conceptive receptive periods (N = 105 female days, 41.7% of all receptive periods; Table 4.2). Of the 35 cycling receptive periods, 17 (N = 86 days) were classified as fertile receptive periods because they overlapped the hormonally defined periovulatory phase. Given that 20 ovulations were identified during the study, we thus detected behavioral receptive periods for 85% of all ovulations.

Eighteen of the 35 cycling receptive periods (61 receptive days) were classified as non-fertile because they occurred outside of the periovulatory phase. Of these 18 non-fertile receptive periods, 47.1% (N = 8, 45.1% receptive days) were found around the time when menstruation would have occurred (the time period overlapping the boundary between the late luteal phase and the early follicular phase). Another 47.1% (N = 8, 45.1% receptive days) were found in the mid-follicular phase, and only 11.1% (N = 2, 9.8% of receptive period days) occurred during the early- to mid-luteal phase. Based on these results, it seems as if the temporal distribution of receptivity was not random, with non-fertile receptive periods occurring mainly around the perimenstrual period and the follicular period.

A substantial portion (N = 11) of the 17 fertile receptive periods also extended into the period preceding, or following the periovulatory period, while only six fell completely within the boundaries of the periovulatory period (Fig. 4.2a). Contrary to the pattern of non-fertile receptive periods, the majority of these extended fertile receptive periods overlapped the luteal period (N = 8 periods, 63.2% of days), although seven of eight of these did not extend more than three days beyond the periovulatory phase (Fig.

4.2a). Taking into account fertile and non-fertile cycling receptive periods, a total of 97 of 147 (66.0%) cycling days constituted non-periovulatory receptive period days. Despite this large percentage, the difference in the number of periovulatory days (6 days) relative to the number of total days (median = 30 days) per ovarian cycle meant that the *likelihood* of a receptivity during days outside of the periovulatory period was still low (Fig. 4.2b). Indeed, when this difference was taken into account, the percentage of female days characterized by receptive periods was much higher around ovulation compared to the rest of the cycle, and was higher during the follicular phase and late luteal period relative to the early-to-mid luteal period.

The likelihood of post-conceptive receptive periods (N = 24) also showed a clear temporal pattern across gestation (Fig. 4.3a). For six females, the first post-conceptive receptive period began between 6-51 days (mean = 29 ± 7.4 days) following the date of conception. However, out of the total 105 receptive period days, only 17.1% occurred prior to day 50 of gestation, while 76.2% occurred between days 50-100. Although only two females were followed substantially past day 100 of pregnancy (Table 4.1), only 7% of all receptive period days were found after this time frame. Furthermore, for the two females who were followed through the last weeks of gestation (B2: 191 days and B6: 197 days; Table 4.1), no receptive periods were observed past day 124.

The distribution of cycling receptive periods suggests that as in other primates, receptive behaviors can occur at any time, but are more likely around ovulation and during the follicular phase. As in other primates (Dixson, 1998), fE levels in Phayre's leaf monkeys increase during the follicular phase to their peak levels during the periovulatory period (Fig. 4.1). A slight increase in receptive behavior perimenstrually is difficult to explain however, as fE levels are generally at their lowest around this time (end of luteal phase to beginning of follicular phase; Fig. 4.1). For post-conceptive receptivity, however, the period of peak receptivity does not seem to be characterized by any clear differences in fE levels compared to the rest of gestation (Fig. 4.3b).

Characteristics of Receptive Periods: Honest Signaling?

Although the temporal pattern of receptivity already suggests that ovulation is not completely concealed, we then looked more specifically at this question, assessing

whether there were differences across different categories of receptive periods in their overall lengths, the lengths of the inter-mating intervals preceding them, and the rates of proceptive and receptive behaviors.

(a) Lengths of the Receptive Period & Inter-mating Interval

The average length of all receptive periods was 4.3 ± 0.5 days (N = 59, median = 4.0 days, range = 1-21 days), with an inter-mating interval of 17.0 ± 1.5 days (N = 46, median = 15, range = 4-44). When we compared all three classes of receptive periods, no significant differences in either receptive period length (Fig. 4.4a; Kruskal Wallis Test, df = 2, H = 3.915, p = 0.141) or the length of the inter-mating interval (Fig. 4.4b: Kruskal Wallis Test, df = 2, H = 4.102, p = 0.129) were found, although closer similarities seemed to exist between fertile (receptive period length: N = 17, mean = 5.1 ± 0.8 days, median = 5.0 days, range = 1-13 days; inter-mating interval: N = 16, mean = 19.9 ± 3.1, median = 16.5, range = 5-44) and post-conceptive receptive periods (receptive period length: N = 24, mean = 4.4 ± 0.9 days, median = 3.0 days, range = 1-21; inter-mating interval: N = 16, mean = 19.9 ± 3.1, median = 16.5, range = 4-39), than between fertile and non-fertile cycling receptive periods (receptive period length: N = 18, mean = 3.4 ± 0.6 days, median = 4.0 days, range = 1-9 days; inter-mating intervals: N = 12, 12.8 ± 2.6 days, median = 10.5 days, range = 4-39).

(b) Rates of Sexual Behavior

Across all receptive periods, proceptive behaviors and receptive behaviors were significantly correlated (N = 59, r = 0.841, $p < 0.001$), occurring at an average rate of 2.22 events/day, and 2.97 events/day respectively. The type of receptive period had a significant effect on the rates of both behaviors (Table 4.3; proceptive: Kruskal Wallis Test: df = 2, H = 18.715, corrected $\alpha = 0.0083$, $p < 0.001$; receptive: Kruskal Wallis Test: df = 2, H = 16.108, corrected $\alpha = 0.0083$, $p < 0.001$). Post-hoc tests showed that the pattern of this difference was also the same for proceptivity and receptivity, with rates of both behaviors higher during fertile receptive periods compared to non-fertile cycling and post-conceptive receptive periods (ns trend for one test; Table 4.4; Fig. 4.5a, 4.5b). The largest difference was found between fertile periods and non-fertile cycling periods, with

the rate of proceptive and receptive behaviors over four and two times more frequent during fertile periods (proceptive: 3.83 vs. 0.70 events/day; receptive: 4.26 vs.1.75 events/day).

Since several of the fertile receptive periods (N = 11) found in this study extended beyond the periovulatory window (Fig. 4.2a), we conducted further post-hoc tests to assess whether rates of behavior were different in the days falling exactly within the window, and those extending beyond the window. Our results show that rates of both proceptive (Wilcoxon Signed Ranks Test: $z = -2.090$, $p = 0.037$, Fig. 4.6a) and receptive behaviors (Wilcoxon Signed Ranks Test: $z = -2.449$, $p = 0.014$, Fig. 4.6b) were higher during days within the fertile window, compared to days outside the fertile window. Proceptive behaviors were nearly 3 times more frequent during periovulatory days (4.53 vs. 1.77 events/day), with the difference in the frequency of receptive behaviors less drastic (5.02 vs. 2.88 events/day). Thus, patterns in rates of proceptivity and receptivity suggest that ovulation is not completely concealed in this species.

Male Patterns: Choice and Confusion?

However, was sexual behavior honest in the case of both males? In order to answer this question, we then investigated whether female proceptive and receptive behavior towards the two breeding males differed. Specifically, we investigated (a) differences in the proportion ("involvement") of fertile, non-fertile cycling, and post-conceptive receptive periods involving each male, (b) differences in rates of proceptivity and receptivity involving each male within each type of receptive period, and (c) differences in the rate of proceptive and receptive behaviors across categories of receptive periods for each male.

(a) Proportion of Receptive Periods

The adult male was involved in nearly all (N = 54, 91.5%) of the 59 total receptive periods, and there was no difference in the pattern of involvement across fertile, non-fertile cycling, and post-conceptive receptive periods (Freeman-Halton Test: $p = 0.221$, Fig. 4.7a). The subadult male, on the other hand, was only involved in 55.9% of total receptive periods, and in contrast to the adult male, there was a significant

association between the type of receptive period, and the percentage of involvement by the subadult male (Freeman-Halton Test: $p = 0.002$, Fig. 4.7b). However, the direction of this association was not entirely as expected. While the subadult was involved in the vast majority (83.3%) of post-conceptive receptive periods, he was involved in only 22.2% of non-fertile cycling receptive periods, and was, in fact, involved in more fertile receptive periods (52.9%) than in non-fertile cycling receptive periods.

(b) Behavioral Rates: Comparisons Within Types of Receptive Periods

When we analyzed which male was more frequently targeted within each class of receptive period (Table 4.3; Fig. 4.8a, 4.8b), we found that as expected, during fertile receptive periods, proceptive and receptive behaviors were more frequently directed at the adult male compared to the subadult male (proceptive: 3.29 vs. 0.36 events/day, Wilcoxon Signed Ranks Test: $N = 18$, $z = -2.722$, corrected $\alpha < 0.0167$, $p = 0.006$; receptive: 3.80 vs. 0.44 events/day, $z = -2.344$, corrected $\alpha < 0.025$, $p = 0.019$). There was also a significant difference between males in the frequency to which they were targets of proceptive (Wilcoxon Signed Ranks Test: $N = 17$, $z = -2.485$, corrected $\alpha < 0.0167$, $p = 0.013$) and receptive ($z = -3.3444$, corrected $\alpha < 0.025$, $p = 0.001$) behaviors during non-fertile cycling receptive periods. However, the direction was not as expected, with the adult male again more frequently involved in both behaviors (proceptive: 0.59 vs. 0.07 events/day; receptive: 1.54 vs. 0.09 events/day). By contrast, during post-conceptive receptive periods, there was a trend for the subadult male to be more frequently involved in both behaviors (proceptive: 1.39 vs. 0.51 events/day, Wilcoxon Signed Ranks Test: $N = 24$, $z = -1.705$, $p = 0.09$; receptive: 1.26 vs. 0.53 events/day, $z = -1.800$, $p = 0.07$).

(c) Behavioral Rates: Within Male Differences Across Receptive Periods

Finally, we looked again across different receptive periods to assess whether the same overall higher rate of sexual behavior in fertile receptive periods relative to non-fertile receptive periods was characteristic of the pattern in each male individually, or only the dominant adult male. Our results support the latter. Although the type of receptive period had a significant effect on the rate of proceptive and receptive behavior

for both males (adult male: Kruskal Wallis Test, proceptive: $H = 19.998$, corrected $\alpha < 0.01$, $p < 0.001$; receptive: $H = 28.423$, corrected $\alpha < 0.01$, $p < 0.001$; subadult male: proceptive: $H = 17.475$, corrected $\alpha < 0.0125$, $p < 0.001$; receptive: $H = 15.014$, corrected $\alpha < 0.0125$, $p = 0.001$), the pattern of difference was not the same for each male (Fig. 4.8a, 4.8b). For the adult male, patterns were much more similar to that found overall (Fig. 4.5a, 4.5b), with rates of both proceptivity and receptivity higher during fertile receptive periods compared to the two other categories of receptive periods (Table 4.5 for details). For the subadult male, rates of proceptivity were higher in post-conceptive compared to fertile receptive periods, and in fertile compared to non-fertile cycling receptive periods. Receptive behavior showed a similar trend; however, the difference between fertile and non-fertile cycling receptive periods was non-significant (Table 4.5; Fig. 4.8a, 4.8b).

When we analyzed differences in behavior within extended fertile periods, we found that the adult male was still the recipient of higher rates of proceptive and receptive behaviors within periovulatory, as opposed to non-periovulatory days (proceptive: Wilcoxon Signed Ranks Test, $z = -2.191$, $p = 0.028$, Fig. 4.9a; receptive: $z = -2.193$, $p = 0.028$, Fig. 4.9b). However, for the subadult male, there were no differences between the two periods in either behavior (proceptive: $z = -0.949$, $p = -0.343$; receptive: $z = -0.405$, $p = 0.686$). Thus, within extended fertile receptive periods, females still seem to favor the adult more when the chances of conception were higher, but show no greater preference for the subadult male.

DISCUSSION

The continuous capacity for receptivity in haplorrhine primates has been hypothesized to be proximately "emancipated" from hormonal control (reviewed in Wallen, 2001), and ultimately related to female strategies of mate choice and paternity confusion (Pawlowski, 1999; van Noordwijk and van Schaik, 2000). Here we presented first data on mating patterns relative to the fertile period in a wild group of Phayre's leaf monkeys.

Likelihood of Receptivity Across the Ovarian Cycle and Gestation

Our analysis of the temporal distribution of receptive periods found that as in many other haplorrhine species, female Phayre's leaf monkeys are receptive throughout the ovarian cycle (Wallen, 2001) as well as during gestation (van Noordwijk and van Schaik, 2000). Within cycling, 51.4% of periods were non-fertile. In addition, 11 of the 17 fertile receptive periods extended outside of the peri-ovulatory window (Fig. 4.2a). Taken together, a total of 66.0% of total cycling receptive period days fell completely outside of the periovulatory period. These numbers suggest that non-fertile mating within cycling females is common in Phayre's leaf monkeys. And indeed, compared to studies on Hanuman langurs in which similar methods for defining receptivity were employed, the percentage of non-fertile receptive period days in cycling females was substantially higher (7.5-13.3% non-fertile receptive days: Borries et al., 2001; Sommer et al., 1992). Note, however, that these studies did not use hormonal measures to define periovulatory periods, and hence, may have underestimated the number of receptive days that were non-fertile.

Furthermore, when we examined the likelihood of receptivity for each day of the ovarian cycle, we found that females were clearly more receptive around ovulation compared to the rest of the cycle (Fig. 4.2b). We therefore suggest that the large total percentage of non-periovulatory receptive days may be due to the overall longer cycle length (complete data set, mean = 38.7 days, median = 30 days, see chapter 5) of the study species. A longer cycle length increases the total number of non-fertile days during which receptivity can occur, but not necessarily the daily likelihood of receptivity outside of the fertile phase. Others have argued that elongating the follicular phase might itself be an adaption to increase female mate choice (Lasley and Benirschke, 1994). However, in this population, longer cycle lengths have been shown to be the result of phytoprogestins, poor nutritional condition, or both (chapter 3). Given the strength of ecological influences, it is difficult to determine whether longer follicular phases have any adaptive value in relation to mate choice.

As in previous studies (e.g., Borries et al., 2001; Loy, 1970; O'Neill et al., 2004), we found that non-fertile receptivity during cycling was more common during the follicular and the late luteal phase, around which menstruation might begin. While

receptivity during the follicular period is likely related to increasing estrogen levels, the hormonal explanation for perimenstrual sexual behavior is unclear. Unlike in humans (Ford and Beach, 1951; Udry and Morris, 1968) and some other catarrhines (e.g., Shimizu et al., 2003), fecal hormone in Phayre's leaf monkeys suggest that a secondary rise in estrogen levels in the late luteal phase is not characteristic of this species (Fig. 4.1; chapter 2). Hence, patterns of this hormone alone cannot explain the greater likelihood of receptivity during this time period (contra Loy, 1970). While we did not present patterns of progesterone in the current paper, it may be that the increased likelihood of sexual behavior around menstruation is related to decreasing progesterone levels (Everitt and Herbert, 1972; Manson, 1986), and/or an increase in the estrogen to progesterone ratio in the late luteal phase. Alternatively, increasing androgen levels have also been proposed to explain perimenstrual receptivity (e.g., Persky et al., 1977). Indeed, the fact that this pattern has been so commonly observed in humans (Udry and Morris, 1968) and other primates (Borries et al., 2001; Kaufmann, 1965; Loy, 1970; Takahata, 1980) suggests that it is regulated by a common physiological mechanism.

As was the case for non-fertile cycling receptive periods, post-conceptive receptive periods also exhibited a clear temporal trend. Specifically, 76.2% of total post-conceptive receptive days occurred between days 50 and 100 of pregnancy, and this period was characterized by the highest percentage of females in receptivity (Fig. 4.3a). This temporal peak in post-conceptive sexual activity mirrors results found in two other Colobine species: Hanuman langurs (*Semnopithecus entellus*: Borries et al., 2001; Sommer et al., 1992) and silvered leaf monkeys (*Trachypithecus cristatus*: Shelmidine et al., 2009), for which sexual behavior also peaks around 50 to 100 days of gestation. Indeed, clear periods of frequent post-conceptive sexual activity characterize many species (e.g., *Cercocebus torquatus*: Gordon et al., 1991; *Leontopithecus rosalia*: Kleiman and Mack, 1977; *Macaca fascicularis*: Engelhardt et al., 2007; *M. mulatta*: Bielert et al., 1976; *Rhinopithecus roxellana*: Yan and Jiang, 2006). However, the time frame marked by peak activity shows variation across taxa (e.g., colobines: day 50-100 of gestation; *Leontopithecus rosalia*: 7-8 weeks prior to birth; *Macaca fascicularis*: day 50-85 of gestation). Although these stereotyped periods of sexual activity again suggest a proximate hormonal basis, in the present study, we did not find that the peak in post-

conceptive mating coincided with any clear temporal difference in fE levels compared to the rest of gestation (Fig. 4.3b). Note, however, that in a previous study (chapter 2, Fig. 2.7), we found that fE levels became consistently higher (N = 6, 79.94 ± 12.56 ng/g feces) than peak follicular phase levels (N = 6, 48.96 ± 2.37 ng/g feces) by the 8th week, or day 50 of gestation. Instead of decreasing again after day 100 however, fE levels remained consistently high throughout late gestation. Therefore, while fE levels might explain the beginning of the peak in post-conceptive receptivity, they fail to explain why receptive behavior is not as common towards the latter half of gestation.

Reduced receptivity during the latter stages of pregnancy is also unlikely to be explained by the estrogen to progestin ratio, as studies on other colobines (He et al., 2001; Ziegler, 2001) show that progestins, just like estrogens, continue to rise throughout the latter half of gestation. The conclusion that estrogen and progestin patterns alone are inadequate at explaining reduced receptivity in late gestation is not novel; similar conclusions have been drawn from two other studies (Bielert et al., 1976; Engelhardt et al., 2007), and suggest that the proximate mechanisms regulating the suppression of post-conceptive mating late in pregnancy require further investigation.

Comparative Receptive Period Length

Our results on receptive period length indicate the average receptive period was 4.3 days long, with fertile receptive periods slight longer, at 5.1 days. This value is much shorter than that found in other species in which females mate promiscuously. For instance, in olive baboons, receptive periods can be 15-20 days long (Smuts, 1985). In contrast, in mountain gorillas, where mating is confined to one male, receptive periods are relatively short, at 1-3 days (Harcourt et al., 1980). Similar patterns were found within Hanuman langurs, for which the mean mid-cycle receptive period length was 7-10 days in multi-male groups (Borries et al., 2001; Heistermann et al., 2001b), but only 4.0 days long in uni-male groups (Sommer et al., 1992). In general, one-male groups are commonly characterized by shorter receptive periods, and this shorter receptivity is hypothesized to result from honest (exact) advertisement of fertility (van Schaik et al., 1999). Longer receptive periods, in contrast, decrease the ability of males to mate guard, thus facilitating paternity confusion. Although the receptive period length of Phayre's

leaf monkeys seems closer to the length of previous studies of one-male groups, this similarity might simply reflect the fact that mating only included two males. Furthermore, despite a shorter receptive period overall and around ovulation, the fact that receptive periods were common outside of the periovulatory period suggests that females still employed some degree of paternity confusion.

Ovulation is Not Completely Concealed in Cycling Females

Indeed, receptive periods were not only common outside of the periovulatory phase, but patterns of receptive period length and the length of the inter-mating interval were not significantly different across different types of receptive periods. Note however, that greater differences seemed to be observed between fertile receptive periods and non-fertile cycling receptive periods. Despite these similarities, rates of proceptivity and receptivity were highest during fertile receptive periods, and specifically in periovulatory days of extended receptive periods. Thus, even though receptivity could occur at any time during the cycle, and several fertile receptive periods (N = 11) overlapped the phase preceeding or following the periovulatory phase, females seemed to convey at least some accurate information on the timing of fertility.

While the majority of primate studies have found that receptivity peaks around ovulation (e.g., Engelhardt et al., 2005; O'Neill et al., 2004; van Belle et al., 2009), the the scale at which the timing of ovulation is known and advertised has varied (Barelli et al., 2008; Borries et al., 2001; Deschner et al., 2003; 2004; Ostner et al., 2006). For example, in long-tailed macaques (*Macaca fascicularis*: Engelhardt et al., 2005), rates of copulations were clearly higher during the periovulatory phase relative to the five days preceding and following this phase. In chimpanzees (*Pan troglodytes*: Deschner et al., 2003; Deschner et al., 2004), on the other hand, copulation rates and sexual swellings both peaked around ovulation, but finer-scale differences between periovulatory days and the days immediately following this period were not significantly different. Similarly, in Hanuman langurs, perimenstrual receptive periods were shorter than mid-cycle receptive periods, and were characterized by a lower proportion of days with copulations (Borries et al., 2001). In addition, copulation rates during conceptive receptive periods were higher compared to non-conceptive receptive periods (Ostner et al., 2006). However,

within fertile receptive periods overlapping the periovulatory phase, copulation rates were highest at the beginning of the receptive period, irrespective of the relative timing of the periovulatory period, suggesting that males could not predict ovulation on a finer scale (Heistermann et al., 2001b).

We found that female Phayre's leaf monkeys were more frequently proceptive and receptive towards males during fertile receptive periods, relative to other periods, and within periovulatory days relative to non-periovulatory days. Hence, it seems that despite being receptive throughout cycling and during gestation, females are providing males with some degree of information, both on a broad level and at a finer level.

Post-conceptive Mating: Manipulating Paternity Assessments?

Interestingly, characteristic differences in receptive periods were not as clear-cut when we compared fertile to post-conceptive receptive periods. Specifically, the average length of the receptive period and the inter-mating interval were closer in length. In addition, while rates of proceptive and receptive behaviors were still higher during fertile receptive periods, the overall difference, at least in terms of proceptivity, was not as extreme (Fig. 4.5a). Similarities between post-conceptive sexual behavior and sexual behavior during cycling have been documented in other species (Barelli et al., 2008; Loy, 1971; 1981) and suggest that at least from a functional perspective, mating during gestation might be used to manipulate male assessments of paternity.

Male Patterns: Female Preference for Subadult Male Only During Gestation

During all cycling receptive periods (fertile and non-fertile), higher rates of proceptive and receptive behavior were directed at the adult compared to the subadult male, and at the adult male during fertile compared to non-fertile receptive periods. However, comparisons both between males and within males suggest that females still preferred the adult male during non-fertile receptive periods. Furthermore, higher rates of behavior for both males (Fig. 4.8a, 4.8b), and a higher percentage of involvement for the subadult male (Fig. 4.7b) during fertile receptive periods suggest that although females might have preferred the adult male overall, they preferred both males (relative to themselves) during periods when the probability of conception was higher. These results

again support the hypothesis that the female strategy underlying non-fertile cycling receptivity included some degree honesty, but might not have been specifically directed at the adult male. Note however, that the preference for the subadult male was no longer significant when we compared periovulatory days relative to non-periovulatory days (Fig. 4.9a, 4.9b).

Once again, post-conceptive mating exhibited a different pattern. Our analyses of the overall pattern of involvement in receptive periods (Fig. 4.7b), rates of behavior between males during different categories of receptive periods, and rates of behavior within males across categories of receptive periods (Fig. 4.8a, 4.8b) all support the same conclusion – that females showed greater preference for the subadult male during post-conceptive receptive periods.

Non-fertile Cycling vs. Post-conceptive Mating: Why the Difference?

Generally speaking, non-fertile mating during cycling and gestation have both been hypothesized to have the same adaptive function: to confuse paternity (van Noordwijk and van Schaik, 2000). Yet in the present study, we found that patterns of mating were not the same for the two, with post-conceptive mating more similar in many respects to fertile mating, and more often directed at the subadult male. These results may suggest that females biased their strategies toward paternity confusion during gestation. One possible explanation for this pattern may be that greater involvement of the subadult male during gestation was a temporal product of maturation. At the beginning of the study, when most females were cycling, females might have employed a mating strategy more conducive to a one-male mating system (see Borries et al., 2001), where individuals are expected to honestly advertise fertility because non-fertile mating would not provide additional benefits. During this time, females may not have directed sexual behavior at the subadult simply because of a lack of interest, or because the costs to mating with the subadult male would be too high if promiscuity provoked aggression (coercion) from the adult male. In the latter half, however, when most females were pregnant and the subadult male was slightly older, an increase in interest and perhaps a decrease in cost might have made it more beneficial for females to employ a strategy tailored more towards paternity confusion. With regards to the latter, it is interesting to note that

although the subadult male was more frequently involved in post-conceptive receptive periods, the adult male harassed relatively few sexual interactions (N = 2, compared to 5 during cycling).

When we actually investigated whether maturation might have had an effect on mating patterns, we found that there was indeed a significant relationship between time of study (first or second half) and subadult involvement in receptive periods (Fisher's Exact Test, $df = 1$, $p < 0.001$). Furthermore, the increase in male involvement characterized both post-conceptive and fertile receptive periods (Fig. 4.10). However, we could not make this comparison for non-fertile cycling receptive periods because cycling females did not exhibit non-fertile receptivity during the latter half of the study. We therefore can conclude that maturity likely had an effect on increasing female interest overall; however we cannot rule out the possibility that the specific differences we found were all due to maturation.

The Function of Post-Conceptive Mating

Post-conceptive mating has been documented in several species of colobines (e.g., *Colobus guereza*: Harris and Monfort, 2006; *Nasalis larvatus*: Gorzitze, 1996; *Rhinopithecus roxellana*: Li and Zhao, 2007; *Semnopithecus entellus*: Borries et al., 2001; Sommer et al., 1992; Yan and Jiang, 2006; *Trachypithecus cristatus*: Shelmidine et al., 2009), and a recent review indicates that it occurs in 59% of all primate species (van Noordwijk and van Schaik, 2000). While commonly considered a counter-strategy to infanticide (Hrdy, 1979; van Noordwijk and van Schaik, 2000), post-conceptive mating is found in species ranging from polyandrous (Kleiman and Mack, 1977) to polygynandrous (Engelhardt et al., 2007), suggesting that the function of such matings might be variable across taxa (Ziegler, 2007). When multi-male mating systems are involved, however, these explanations generally depend on the idea that post-conceptive sexual behavior is a female adaptation to confuse paternity in order to acquire some benefit in return.

Although infanticide is thought to be common among colobines (*Colobus guereza*: Harris and Monfort, 2003; Oates, 1977; Onderdonk, 2000; *Colobus vellerosus*: Teichroeb and Sicotte, 2008; *Presbytis senex*: Rudran, 1973b; *Presbytis thomasi*: Steenbeck, 2000; *Rhinopithecus bieti*: Xiang and Grueter, 2007; *Semnopithecus entellus*:

Hrdy, 1974; Borries, 1997) and in primates in general (van Schaik, 2000a), it has never been observed in Phayre's leaf monkeys, despite eight years of research. Furthermore, observations of females visiting other groups with dependent infants in tact suggest infanticide is not common in the population. One possible explanation for this pattern is that the incentive for males to commit infantide has been lowered due to female sexual strategies. By extending receptivity and mating promiscuously, females successfully confuse paternity, thereby reducing the risk of infanticide because males are unsure if they would be killing their own infants. Indeed, paternity confusion might even work across groups, since females often visit neighboring groups, and extra-group paternity has been documented in the population (Larney, unpublished data).

In addition to countering infanticide, it is also possible that females mate post-conceptively in return for some other future benefit (Rodriguez-Girones and Enquist, 2001; Soltis, 2002). Male Phayre's leaf monkeys regularly affiliate with immatures (Koenig et al., 2009), even occasionally carrying them. Males also intervene in female conflicts (Koenig & Borries, unpublished data). Whether these actions are indeed services "paid" in return for mating opportunities can only be answered with future studies comparing non-fertile mating patterns and male behavior towards females and immatures.

Male Knowledge

Regardless of the specific adaptive function of paternity confusion, females are expected to target subordinate males during post-conceptive mating. Because dominant males generally have high paternity assurance, they are unlikely to commit infanticide. Furthermore, dominant males have greater access to females, particularly during peak fertility, so are unlikely to offer additional benefits in return for yet more mating opportunities (Gangestad and Thornhill, 2008; Rodriguez-Girones and Enquist, 2001). It is therefore not surprising that a preference for mating with subadults has been found in numerous other studies (Carnegie et al., 2005; Engelhardt et al., 2007; Gordon et al., 1991; Gust, 1994; but see Inoue et al., 1993).

For paternity confusion to be successful, however, males must be unable to discriminate between non-fertile and fertile matings. In the present study, shared

characteristics between fertile receptive periods and post-conceptive receptive periods suggest that this might have been the case. Furthermore, males seemed to respond to behavioral cues, since rates of proceptivity were strongly correlated with receptivity. As noted previously however, despite the high number of post-conceptive receptive periods involving the subadult, the adult male was only observed to harass matings twice during this time. These results suggest that either the adult male had some additional way to discriminate fertility (Gust, 1994), or both males could equally discriminate fertility (Carnegie et al., 2005). Indeed, as part of the "arms race" in sexual selection, males are expected to evolve methods to counter-act female strategies. If such an interpretation is correct, however, the subordinate male would have copulated with females for reasons unrelated to direct fitness benefits. Instead, mating with females during a period with zero probability of conception might have been more generally related to bonding (Gust, 1994). Future analyses focusing on female attractivity will give us more insight into the degree to which males can discriminate female fertility, and hence, provide more clues as to the reasons why males participate in post-conceptive mating.

Table 4.1 Conception dates, number of days that females were followed when cycling and pregnant, and number of fecal samples collected during cycling and gestation (N = 7 females).

Female	Conception Date	N Days Cycling	N Days Pregnant	N Fecal Samples Cycling	N Fecal Samples Pregnant
B2	March 21, 2006	48	191	38	136
B3	June 6, 2006	125	115	102	99
B5	June 8, 2006	127	114	117	101
B6	March 18, 2006	45	197	49	147
B7	September 7, 2006	218	25	66	19
B9	May 24, 2006	112	132	96	101
B12	June 27, 2006	146	99	135	71

Table 4.2 Distribution of fertile receptive periods, non-fertile cycling receptive periods, and post-conceptive receptive periods (N receptive periods, N total female days).

Category	N periods	% Total periods	N female days	% Female days
Fertile	17	28.9%	86	34.1%
Non-fertile cycling	18	30.5%	61	24.2%
Post-conceptive	24	40.6%	105	41.7%

Table 4.3 Rates of proceptive and receptive behavior during fertile, non-fertile receptive, and post-conceptive receptive periods. Data (events/day) presented for overall behavior, and behavior directed specifically at the adult male and the subadult male.

Category	Involvement	Fertile	Non-fertile Cycling	Post-conceptive
Proceptive	Overall	3.83	0.70	2.01
	Adult male	3.29	0.59	0.51
	Subadult male	0.36	0.07	1.39
Receptive	Overall	4.26	1.75	1.85
	Adult male	3.80	1.54	0.53
	Subadult male	0.44	0.09	1.26

Table 4.4 Post-hoc comparisons of differences in rates of proceptive and receptive behavior across three different types of receptive periods.

Category	Comparison	U	Z	P value	Significance
Proceptive	Fertile vs. Non-fertile Cycling	30.0	-4.068	<0.001	*
	Fertile vs. Post-conceptive	136.0	-1.804	0.071	ns trend
Receptive	Fertile vs. Non-fertile Cycling	43.0	-3.665	<0.001	*
	Fertile vs. Post-conceptive	95.0	-2.900	0.004	*

Table 4.5 Post-hoc comparisons of differences in rates of proceptive and receptive behavior across three different types of receptive periods within each male separately.

Male	Behavior	Post-hoc Comparison	U	z	p value	Significance
M5	Proceptive	Fertile vs. Non-fertile Cycling	42.0	-3.684	<0.001	*
		Fertile vs. Post-conceptive	50.0	-4.098	<0.001	*
	Receptive	Fertile vs. Non-fertile Cycling	63.5	-2.991	0.003	*
		Fertile vs. Post-conceptive	23.5	-4.815	<0.001	*
m12.1	Proceptive	Fertile vs. Non-fertile Cycling	106.5	-1.988	0.046	*
		Fertile vs. Post-conceptive	118.0	-2.347	0.019	*
	Receptive	Fertile vs. Non-fertile Cycling	112.0	-1.687	0.090	ns trend
		Fertile vs. Post-conceptive	125.5	-2.516	0.031	*

Figure 4.1 Composite graph of fE levels in 20 cycles relative to the day of the fE peak (mean ± SEM; shaded region = defined periovulatory phase of the cycle; dotted line = estimated ovulation date).

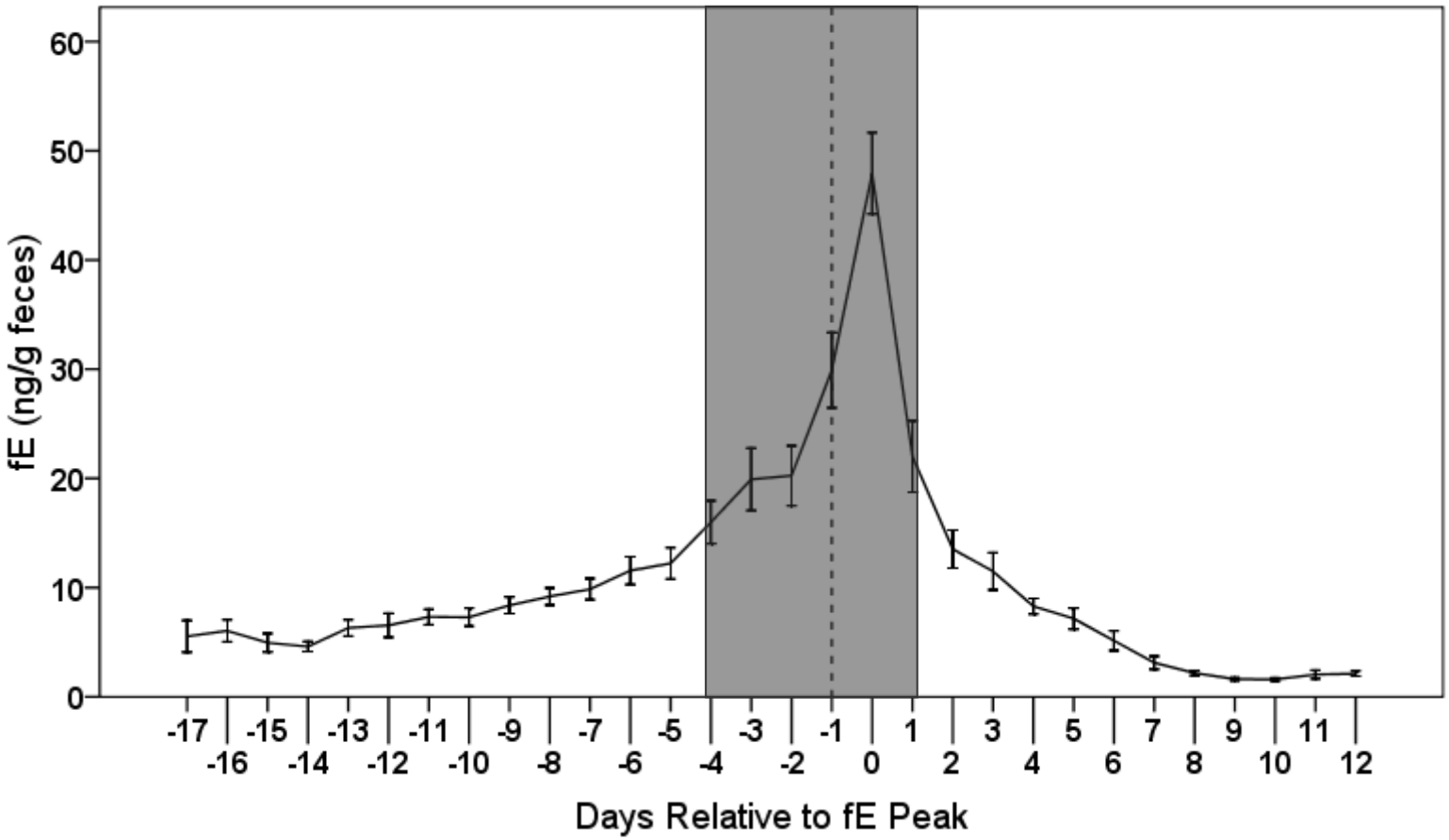


Figure 4.2a Distribution of fertile receptive periods relative to the defined six-day periovulatory phase of the cycle (N = 17 cycles; gray = periovulatory period; black = fertile receptive periods; hatched = non-fertile cycling receptive periods; dotted line brackets = estimated ovulation date; asterisks = conceptive cycles; number of days outward from the fE peak dependent on length of the follicular and luteal phases in individual cycles).

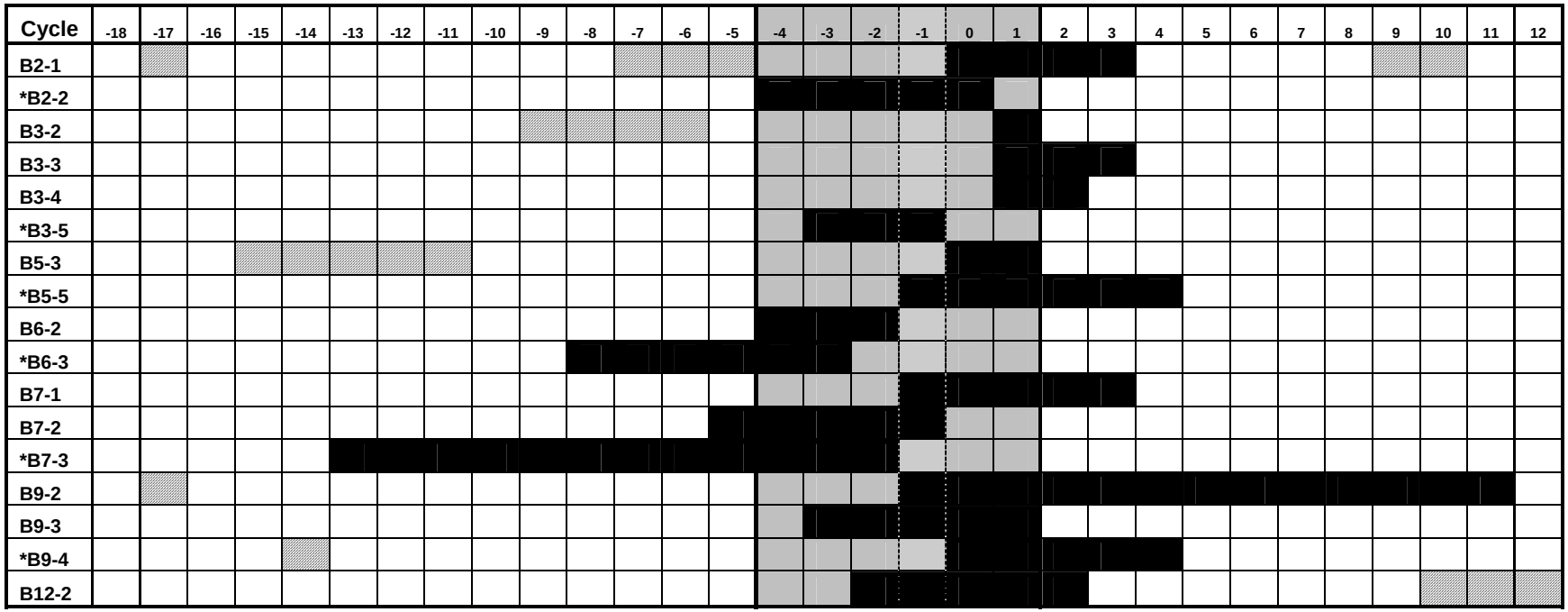


Figure 4.2b Percentage of female days during ovarian cycles falling within defined periods of receptivity (N = 20 cycles; gray = periovulatory period; dotted line = estimated ovulation date; number of days outward from the fE peak dependent on length of the follicular and luteal phases in individual cycles).

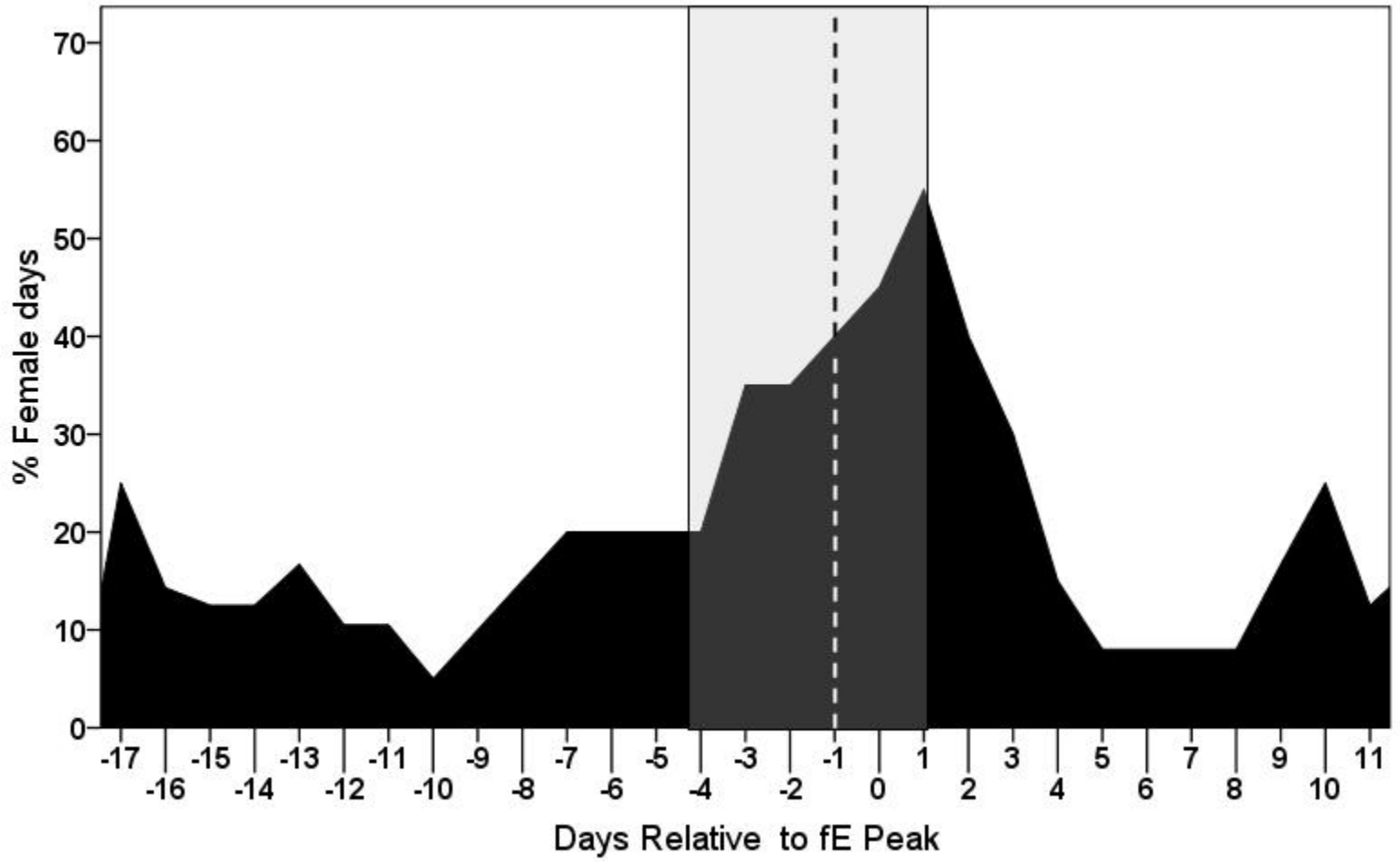


Figure 4.3a Percentage of female days during gestation that fall within defined periods of receptivity (N = 6 females). Note that after day ~115, the profile is only represented by two pregnancies.

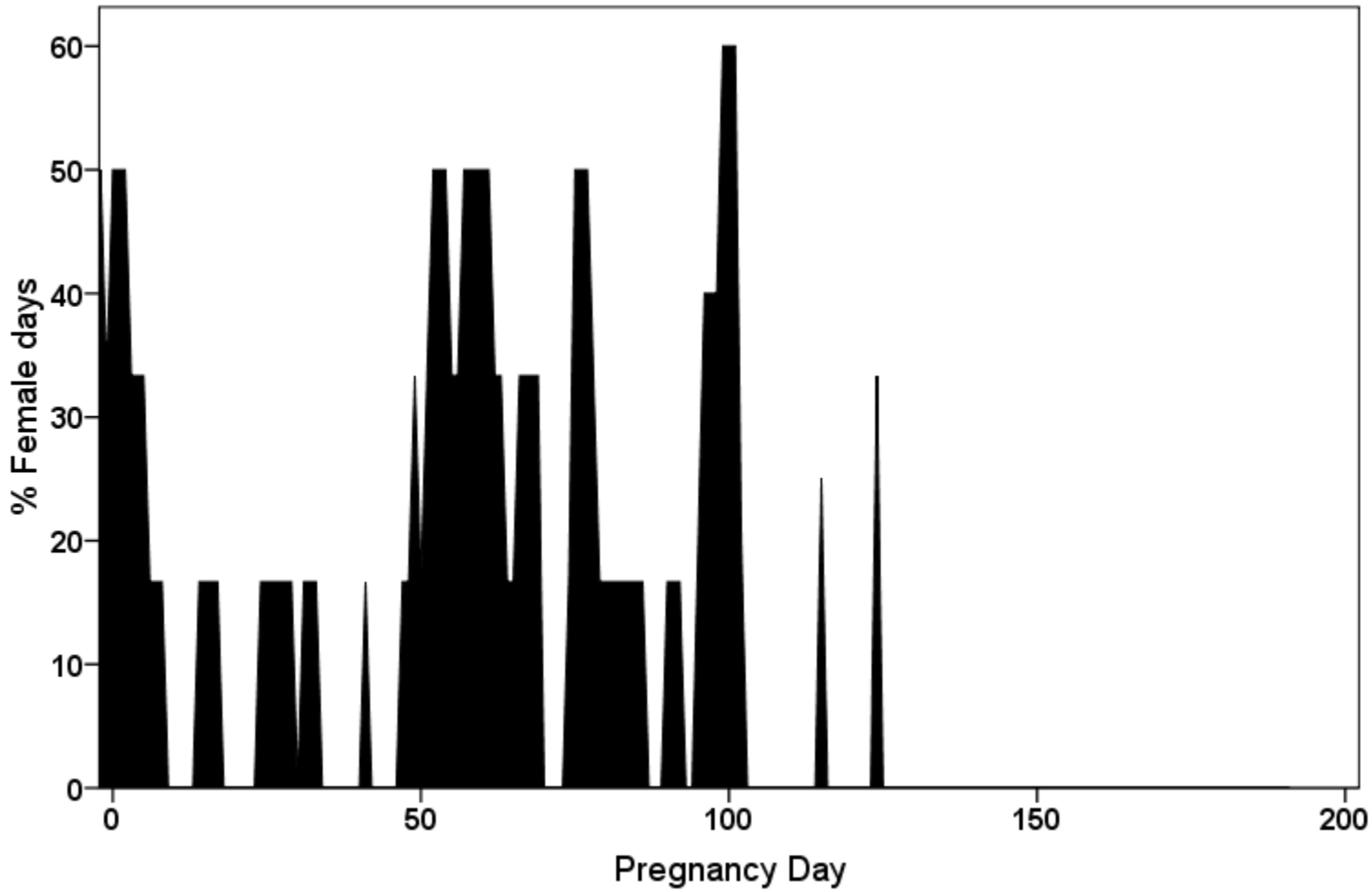


Figure 4.3b Patterns of fE levels averaged every five days throughout gestation (N = 6 females; mean ± SEM; shaded region = days 50-100, when receptivity was most common). Note that after day ~115, the profile is only represented by two pregnancies.

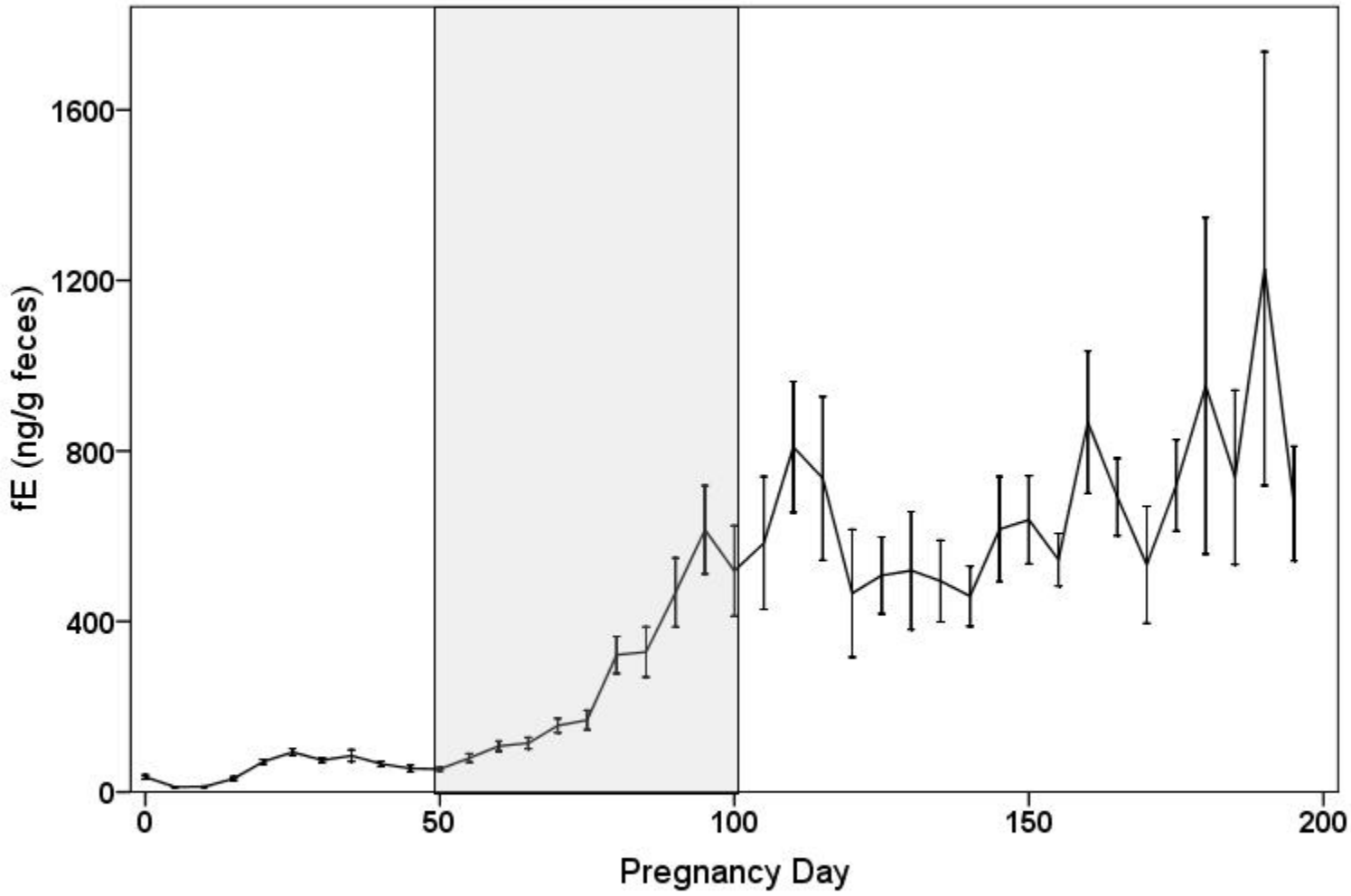


Figure 4.4a Length of non-fertile cycling, fertile, and post-conceptive receptive periods (solid line = median, box = interquartile range, error bars = 95% confidence intervals). No significant differences between groups (Kruskal Wallis Test, df = 2, H = 3.915, p = 0.141).

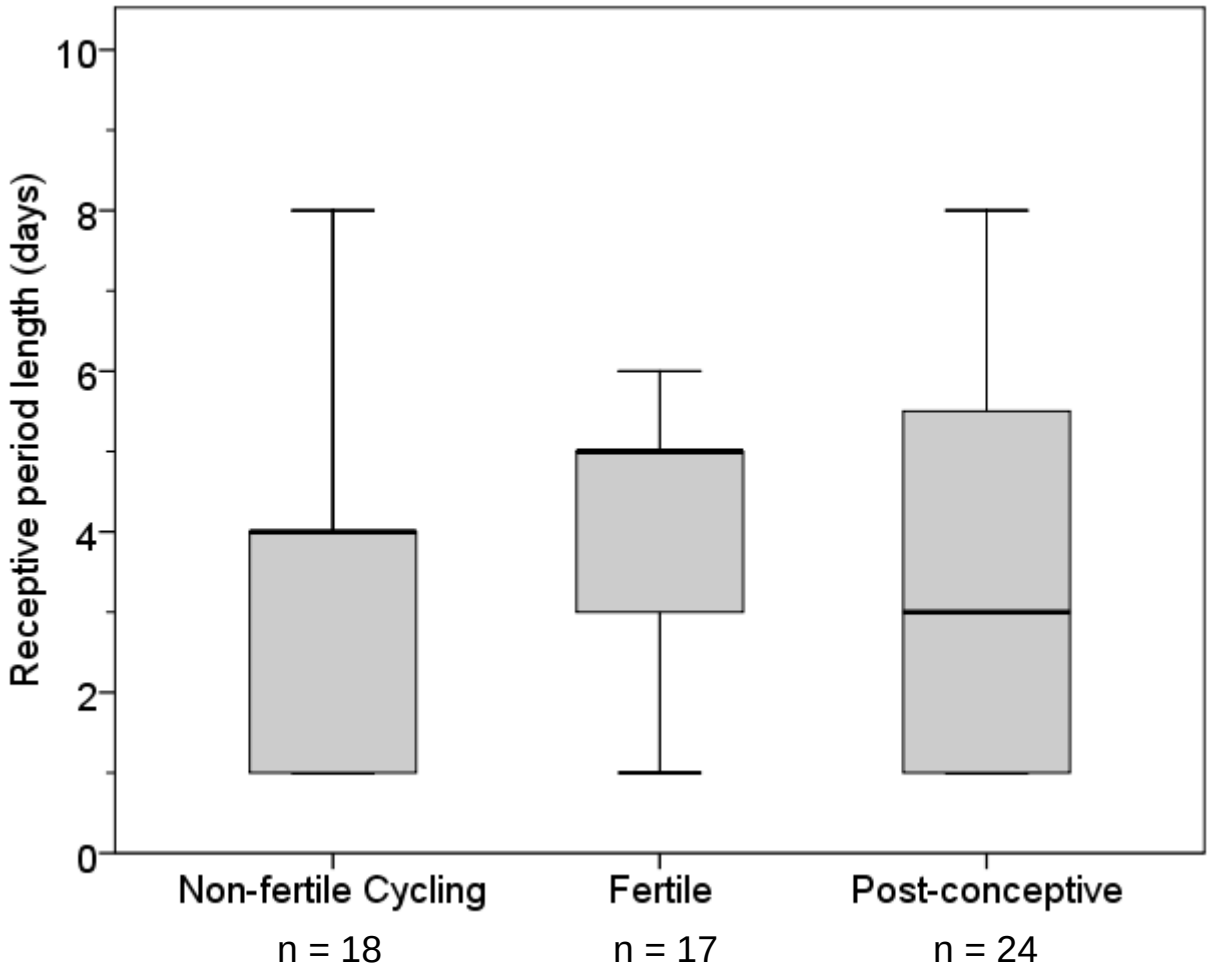


Figure 4.4b Length of the inter-mating interval preceding non-fertile cycling, fertile, and post-conceptive receptive periods (solid line = median, box = interquartile range, error bars = 95% confidence intervals). No significant differences between groups (Kruskal Wallis Test, df = 2, H = 4.102, p = 0.129).

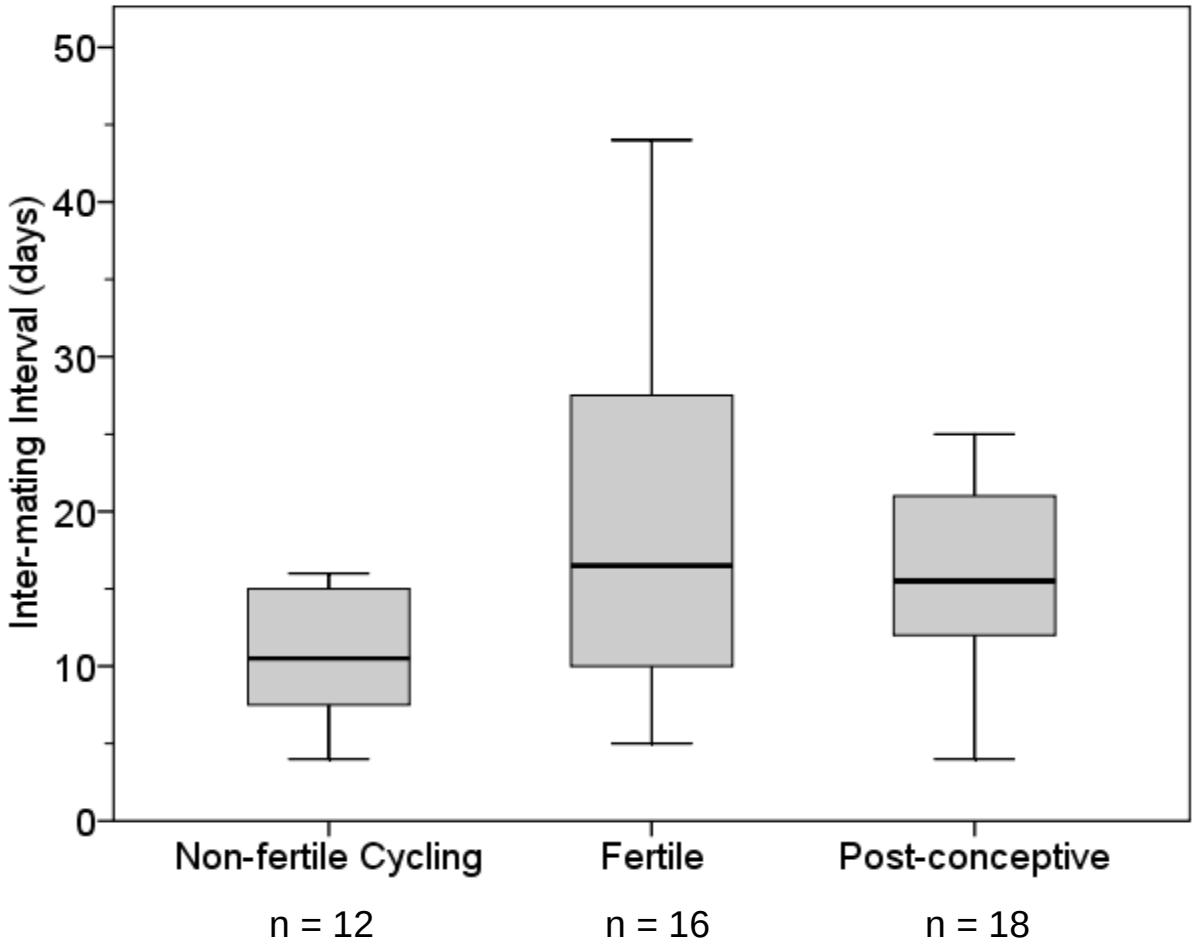


Figure 4.5a Rates of proceptivity during non-fertile cycling, fertile, and post-conceptive receptive periods (mean ± SEM). Differences between fertile and non-fertile cycling receptive periods (Mann Whitney U Test: U = 30.0, z = -4.068, corrected $\alpha < 0.025$, $p < 0.001$, significant (*)) and fertile and post-conceptive receptive periods (U = 136.0, z = -1.804, p = 0.071, ns trend).

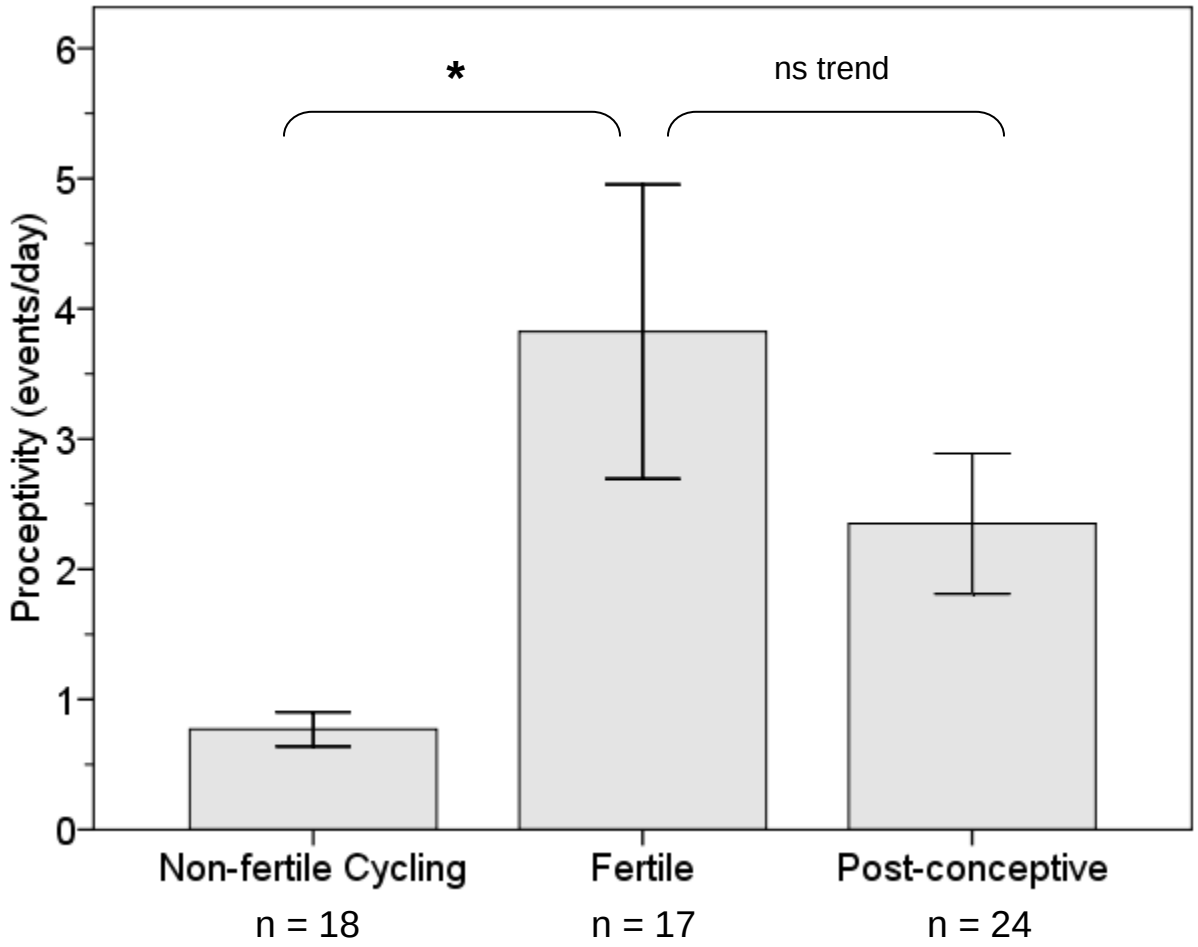


Figure 4.5b Rates of receptivity during in non-fertile cycling, fertile, and post-conceptive receptive periods (mean ± SEM). Significant (*) differences between fertile and non-fertile cycling receptive periods (Mann Whitney U Test: U = 43.0, z = -3.665, corrected $\alpha < 0.025$, $p < 0.001$) and fertile and post-conceptive receptive periods (U = 95.0, z = -2.900, corrected $\alpha < 0.025$, $p = 0.004$).

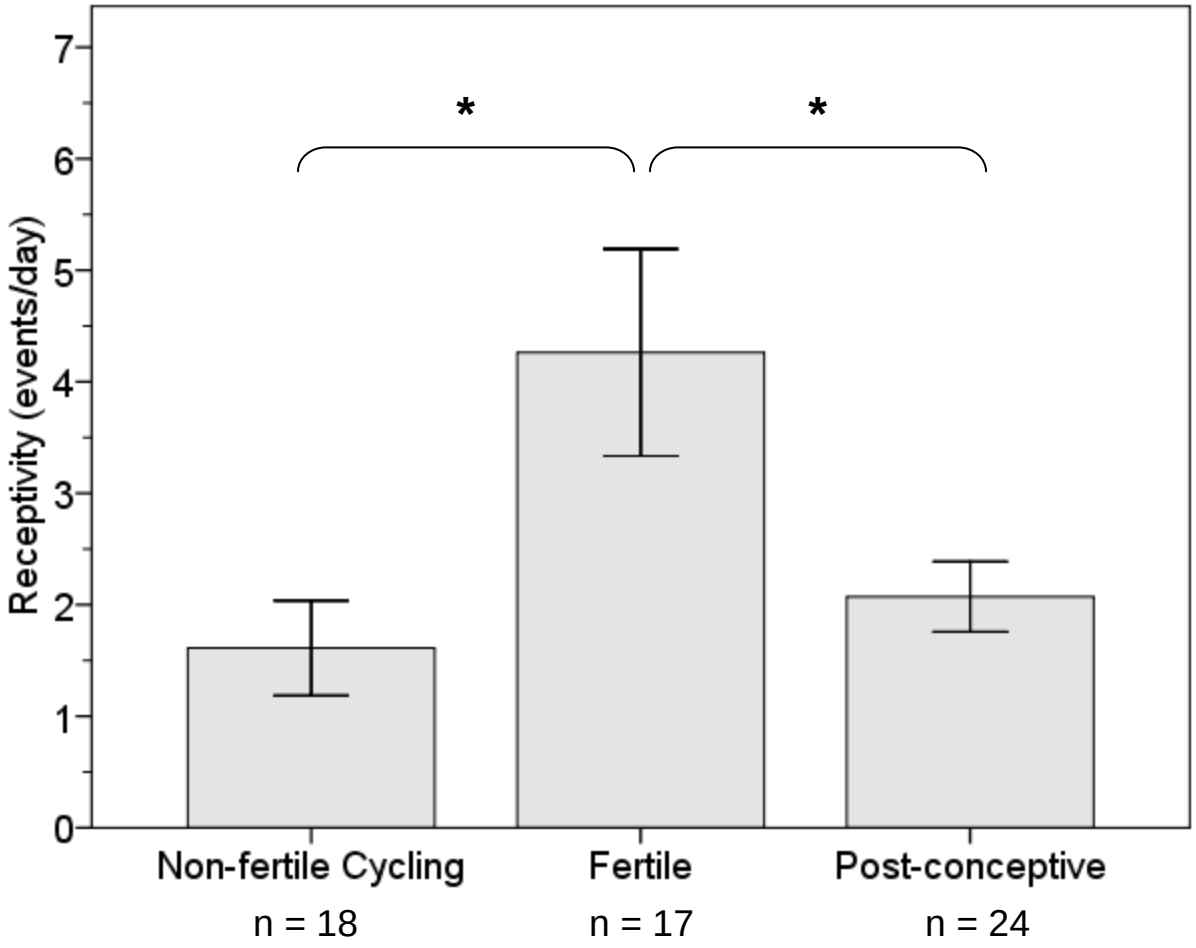


Figure 4.6a Rates of proceptivity during periovulatory and non-periovulatory days within extended fertile receptive periods (mean ± SEM). Significant (*) difference (N = 11; light dotted = periovulatory, gray dotted = non-periovulatory; Wilcoxon Signed Ranks Test: z = -2.090, p = 0.037).

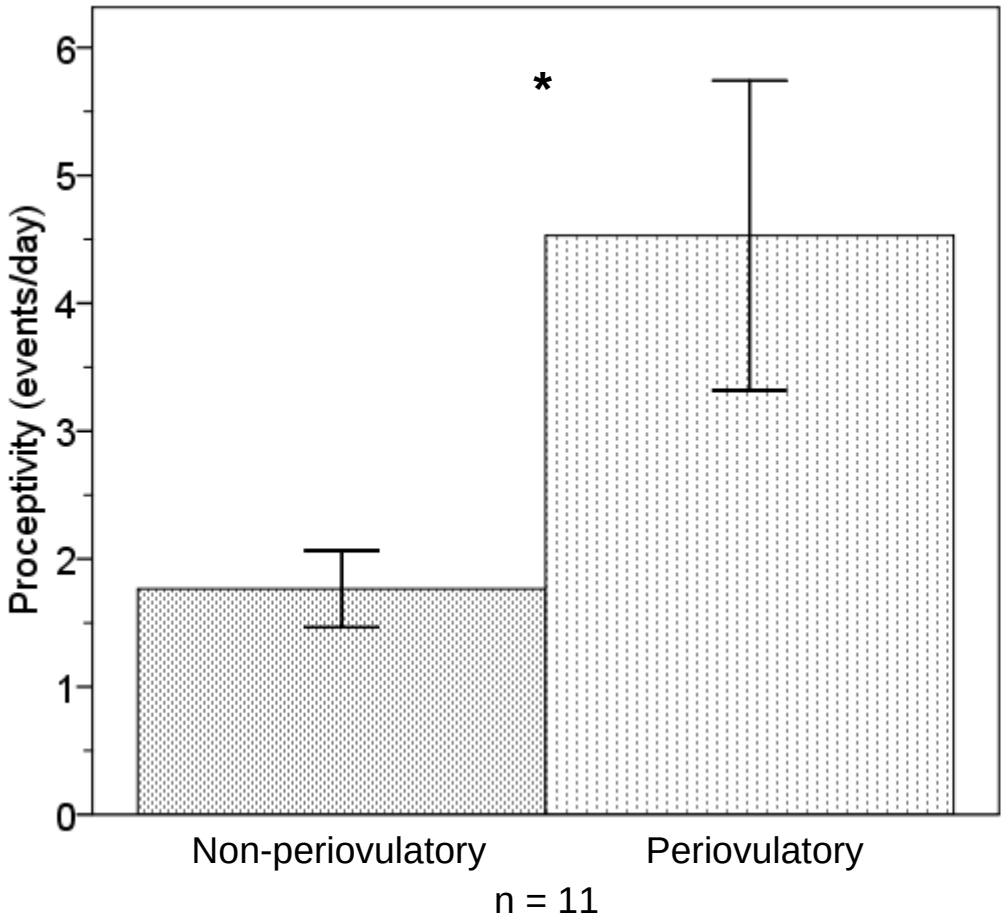


Figure 4.6b Rates of receptivity during periovulatory and non-periovulatory days within extended fertile receptive periods (mean ± SEM). Significant (*) difference (N = 11; light dotted = periovulatory, gray dotted = non-perovulatory; Wilcoxon Signed Ranks Test: z = -2.449, p = 0.014).

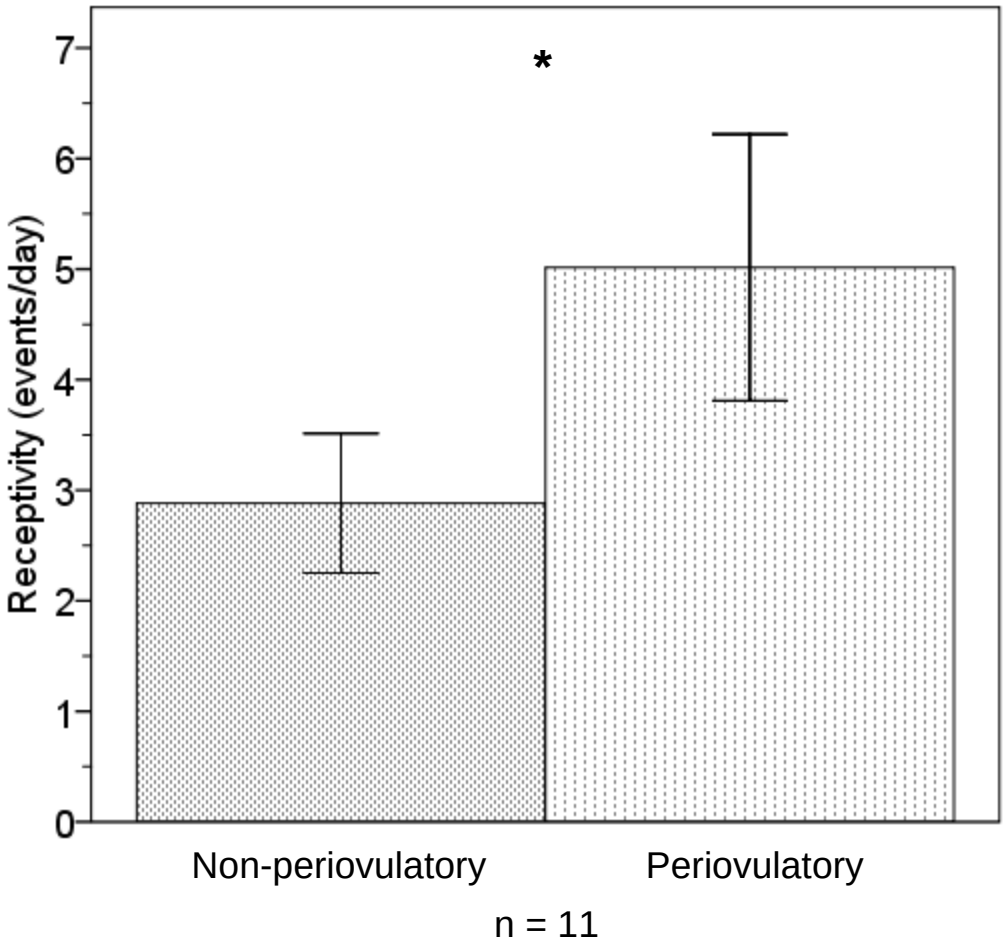


Figure 4.7a Proportion of adult male involvement in non-fertile cycling, fertile, and post-conceptive receptive periods. Significant relationship between involvement and category of receptive period (Freeman-Halton Test: p = 0.221).

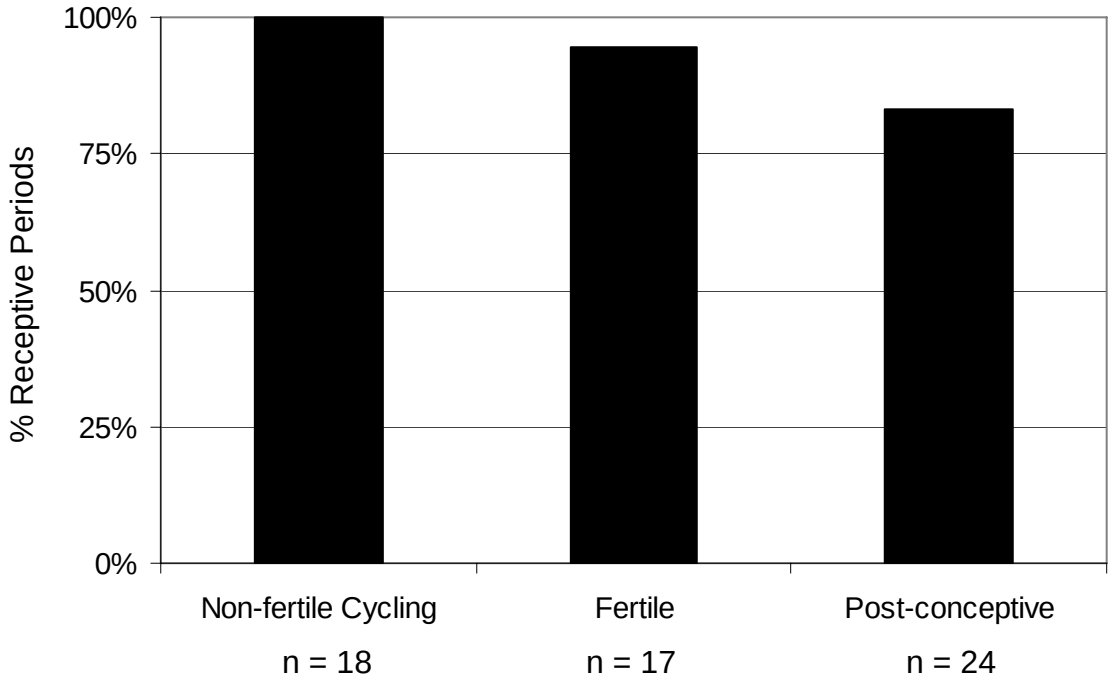


Figure 4.7b Proportion of subadult male involvement in non-fertile, fertile, and post-conceptive receptive periods. Significant relationship between involvement and category of receptive period (Freeman-Halton Test: p = 0.002).

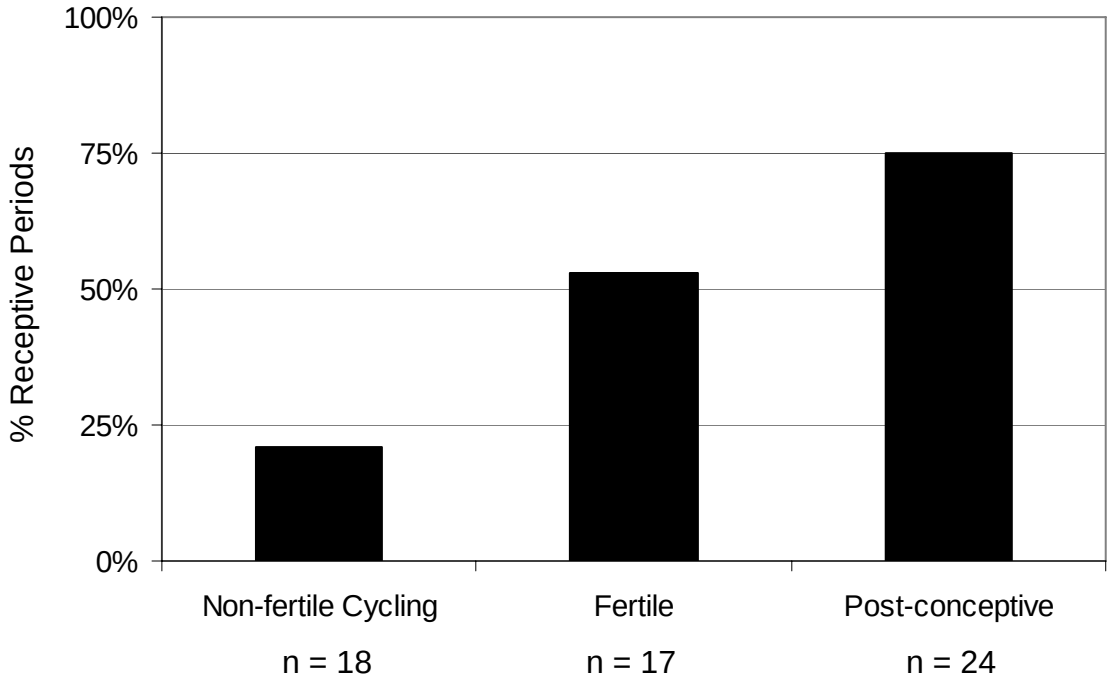


Figure 4.8a Rates of proceptivity involving the adult male and the subadult male during non-fertile cycling, fertile, and post-conceptive receptive periods (mean ± SEM; adult male = gray bars; subadult male = white bars). All differences between males (Wilcoxon Signed Ranks Test, corrected $\alpha < 0.05$) and within males (Mann Whitney U Test, corrected $\alpha < 0.05$) significant, except non-significant trend ($z = -1.705$, $p = 0.09$) between males in post-conceptive receptive periods.

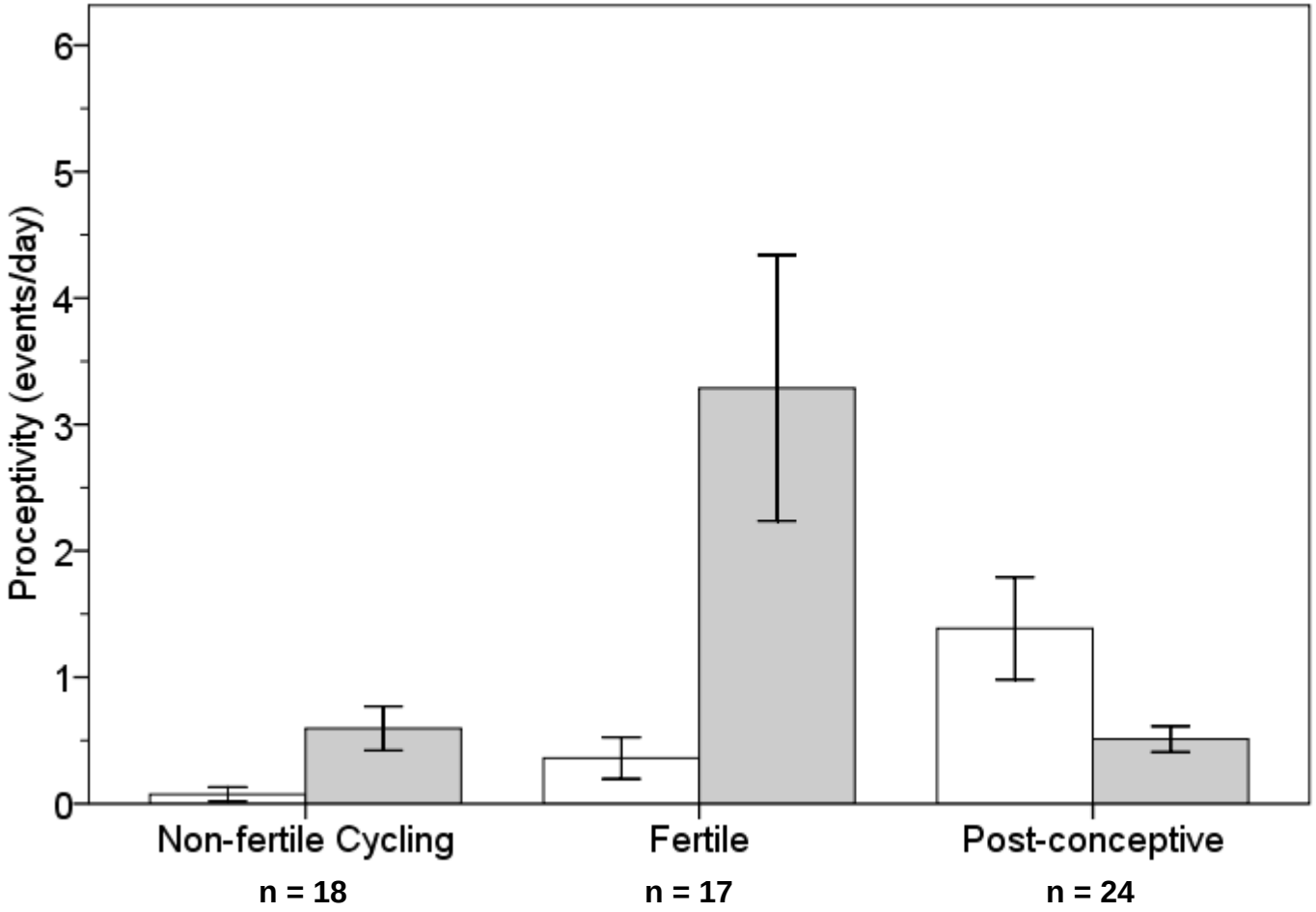


Figure 4.8b Rates of receptivity involving the adult male and the subadult male during non-fertile cycling, fertile, and post-conceptive receptive periods (mean ± SEM; adult male = gray bars; subadult male = white bars). All differences between (Wilcoxon Signed Ranks Test, corrected $\alpha < 0.05$) and within males (Mann Whitney U Test, corrected $\alpha < 0.05$) significant except for non-significant trend between males in post-conceptive receptive periods ($z = -1.800$, $p = 0.07$), and within the subadult male, between fertile and non-fertile cycling receptive period ($U = 112.0$, $z = -1.687$, $p = 0.09$).

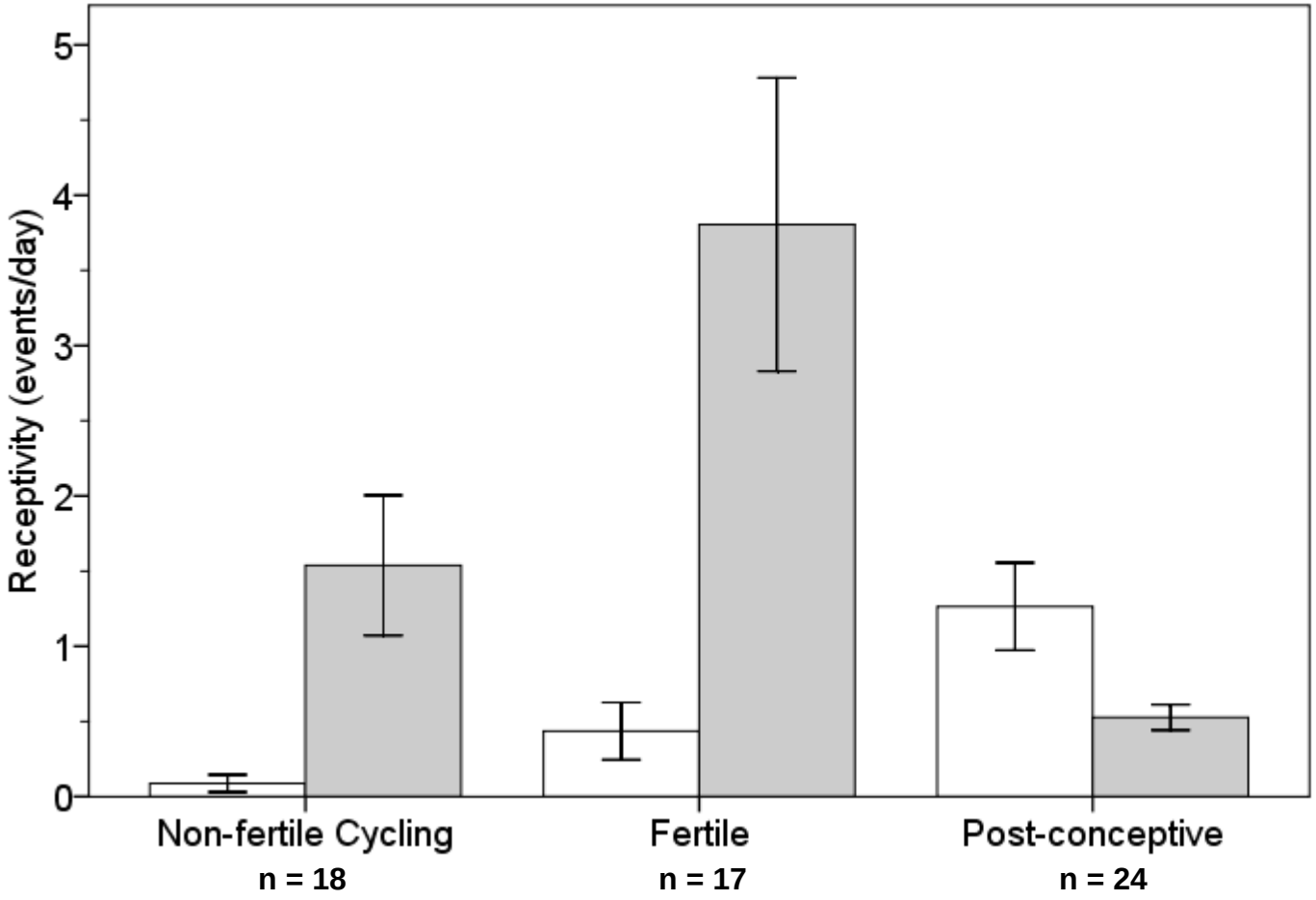


Figure 4.9a Rates of proceptivity in the adult and subadult male during periovulatory and non-periovulatory days within extended fertile receptive periods (mean ± SEM; light dotted = periovulatory, gray dotted = non-periovulatory). Significant (*) difference for adult male only (Wilcoxon Signed Ranks Test, z = -2.191, p = 0.028).

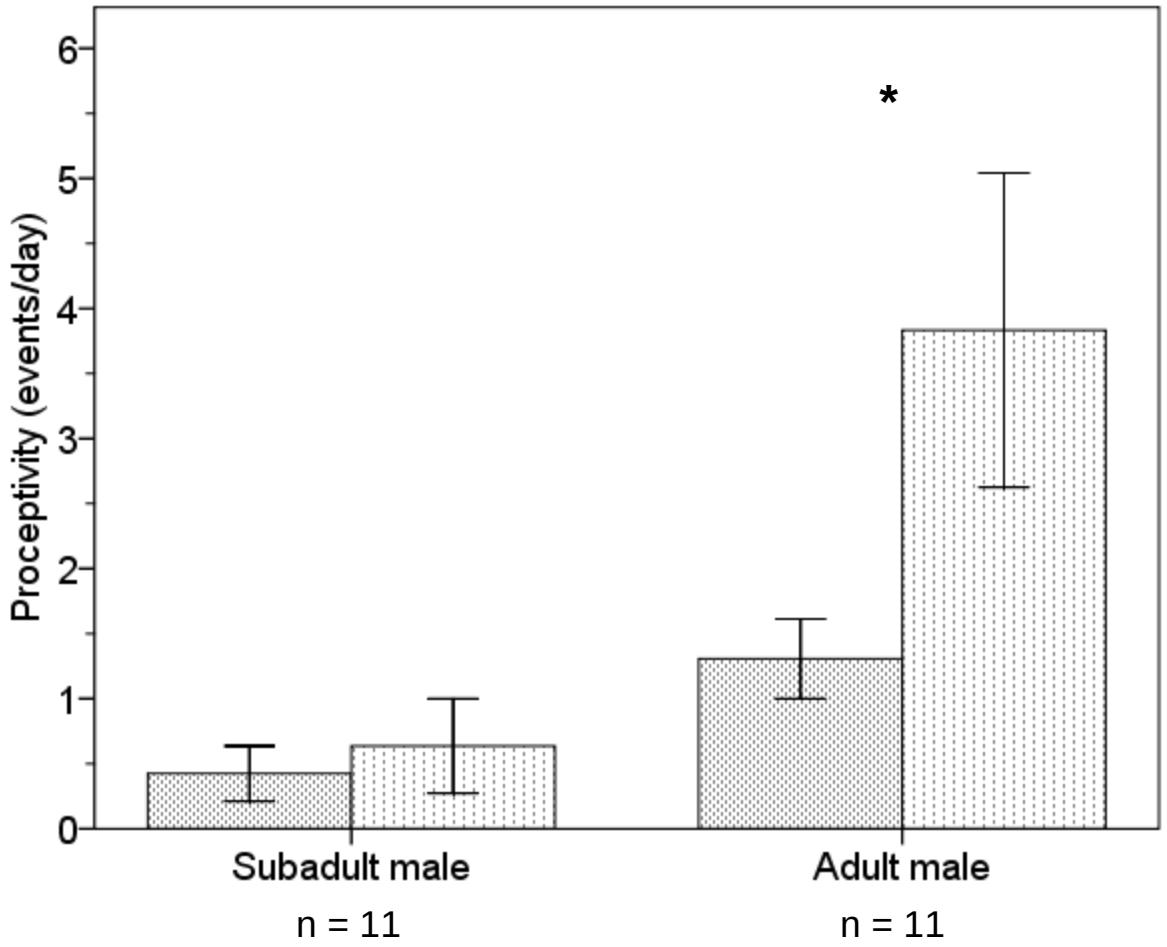


Figure 4.9b Rates of receptivity in adult male and subadult male during periovulatory and non-ovulatory days within extended fertile receptive periods (mean ± SEM; light dotted = periovulatory, gray dotted = non-periovulatory). Significant (*) difference for adult male only (Wilcoxon Signed Ranks Test, z = -2.193, p = 0.028).

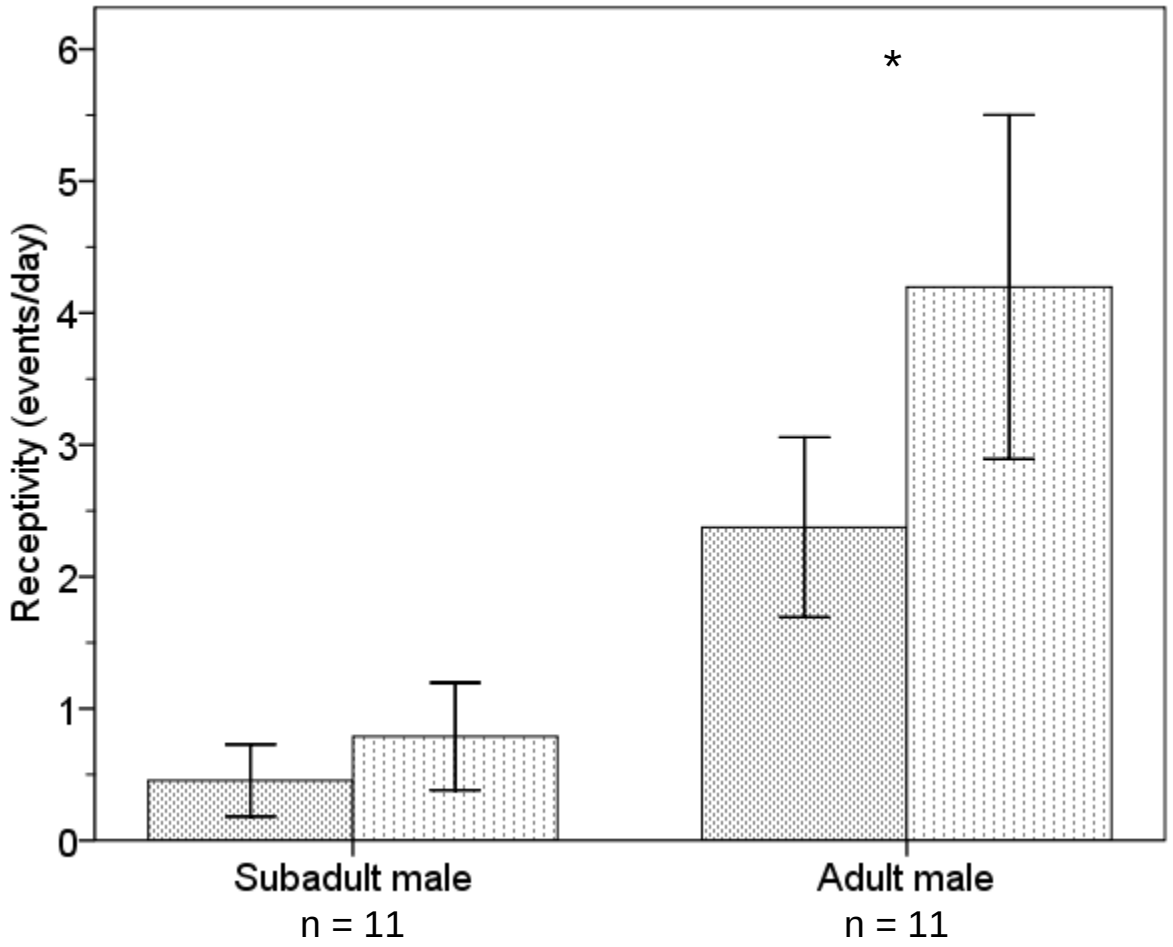
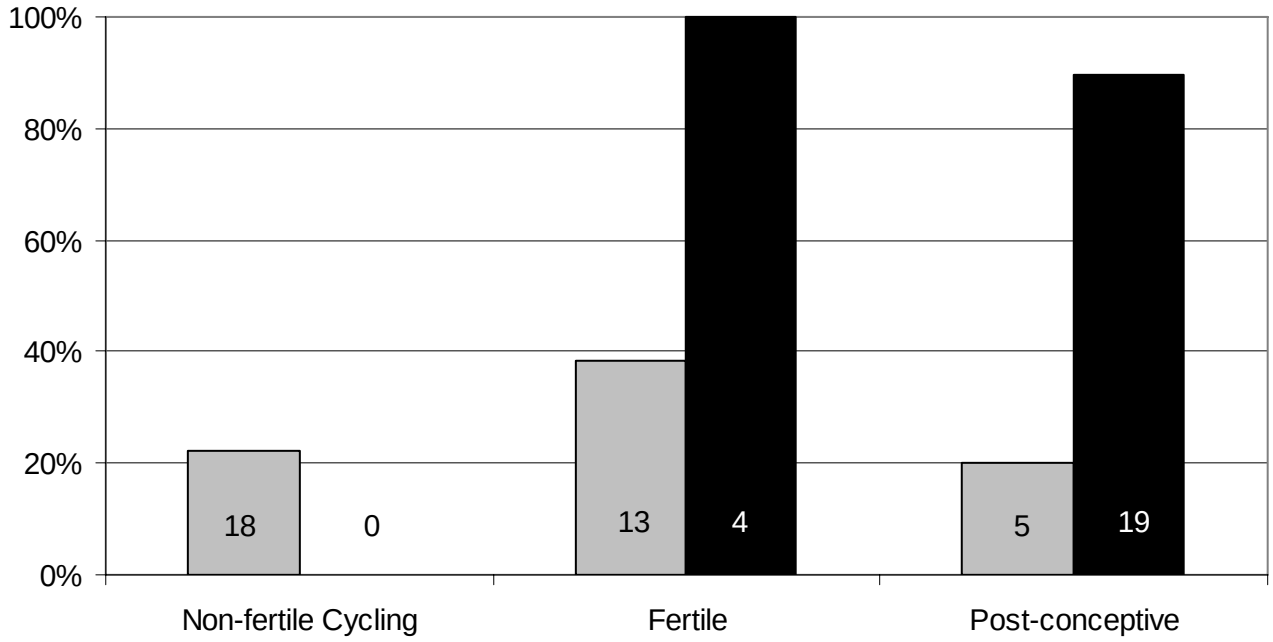


Figure 4.10 Proportion of involvement of the subadult male in receptive periods during the first four months of the study (February – May, 2006), relative to the last four months (June – September, 2006) for different categories of receptive periods (gray bar = first four months; black bar = last four months; total number of receptive periods out of which percentages were based are noted for each bar).



CHAPTER FIVE

REPRODUCTION AND MATING PATTERNS IN PHAYRE'S LEAF MONKEYS: SYNTHESIS OF FINDINGS AND PROPOSAL FOR FUTURE DIRECTIONS

In this study, the first results on female reproductive parameters, ecological factors shaping female reproduction, and female mating patterns and mate preference were presented in a wild group of Phayre's leaf monkeys. The study was only the third to use fecal steroid monitoring to monitor reproduction in a wild colobine species, and the first to do so in the genus *Trachypithecus*. In the following section, I provide a synthesis of our results, and suggest directions for future research. I focus both on methodological and theoretical issues. To avoid repetition, greater emphasis is placed issues not yet discussed in previous chapters.

NON-INVASIVE STEROID MONITORING: OPTIMIZATION OF PROTOCOLS

One of our main accomplishments was the successful validation of the use of fecal steroids to document reproductive characteristics in Phayre's leaf monkeys. Specifically, we were able to: (1) assess conception and the beginning of the luteal phase based on fecal progestin metabolites for part of the study period, (2) determine seasonal patterns in excreted progestins and compare these to the availability of *Vitex*, and (3) identify both ovulation and the fertile period, and assess nearly all parameters related to cycling and gestation, based on patterns of estrogen metabolites. In fact, in this study, the pattern of fecal estrogen excretion was a more useful tool for documenting reproductive function. This was due to two reasons: (1) the inadequacy of fecal progestin values to delineate the end of the luteal phase, and (2) the presence of dietary phytoprogestins that obscured fecal progestin patterns related to reproduction for the latter part of the study.

Although the second of these issues could not have been avoided, the first might have been circumvented, had we assessed the efficacy of several different progestin antibodies in documenting reproductive patterns in the study species (e.g., Heistermann et al., 1995; Heistermann et al., 2006; Wasser et al., 2000). Primates show great variation in

steroid metabolism (Bahr et al., 2000), and the antibody that most successfully tracks reproductive function may vary tremendously from species to species (e.g., Heistermann et al., 2006 for comparison of glucocorticoid Ab). However, comparative laboratory analyses are costly, and more importantly, are dependent on the accessibility of assay protocols, the materials that are required to run them, and any results, published or unpublished, on the success and failures of such protocols. Future studies, therefore, would greatly benefit from a shared database of assay protocols that have been applied to various different species of primates. With such information, researchers will be better equipped to develop and optimize new and existing protocols.

REPRODUCTIVE PARAMETERS: SPECIES-SPECIFIC VALUES AND COMPARATIVE ANALYSES

Based mainly on patterns of estrogens, we provided the first estimates of gestation length, cycle length, and the follicular and luteal portions of the cycle in female Phayre's leaf monkeys. In general, our results for gestation length and menstrual cycle parameters were consistent with previous studies on colobines. However, the species-typical values we found for cycle length and the length of the follicular phase were much lower and less variable than that found when data from the entire study period were included (Table 5.1). Our analysis of the effects of phytoprogestins on reproduction (chapter 3) supported the idea that elongated cycle lengths and follicular phases in many of cycles were due to the effect of ecology. Specifically, we found mean cycle and follicular lengths during the normal progestin period were much lower compared to the high progestin period (Table 5.1).

Despite this confirmation, our results suggest that even when we controlled for the effect of phytoprogestins (Table 5.1, normal progestin period), cycle lengths and follicular lengths remained longer and substantially more variable compared to those found in previous colobine studies. Rather than invoking additional ecological or evolutionary factors to explain this difference, we suggest that the longer and more variable lengths were due to the inclusion of data from an anomalous female, B12, who was characterized by the longest cycle lengths, both during the high progestin period (59 days), and the normal progestin period (90 days). Indeed, the species typical values we

produced were not only based strictly on cycles during the normal progestin period, but did not include values from B12, as these were considered statistical outliers (chapter 2, Table 5.1).

Accurate species-typical averages are crucial to comparative analyses. On the one hand, if the purpose of comparative analysis is to investigate factors influencing variation across populations within closely related taxa (e.g., Borries et al., 2001; Hill and Dunbar, 2003; Hill et al., 2000), averages that reflect ecological variation are pertinent to the question, and therefore should be included in the analysis. However, when evolutionary (Barnett and Abbott, 2003) or allometric relationships (Martin and MacLarnon, 1985) are the main focus, averages that either (1) reflect current ecological variation, or (2) include individual anomalies are less likely to yield clear results, as they include additional statistical "noise." For instance, it has often been hypothesized that longer and more variable follicular phases (compared to the luteal phase) have evolved to facilitate paternity confusion and mate choice in primate females (Lasley and Benirschke, 1994; van Schaik et al., 2000). Although all primates generally show this pattern (van Schaik et al., 2000), it might be fruitful to evaluate whether species exhibit differences in the ratio of luteal phase to follicular phase variation and whether this variation is related to some factor such as the lactation to gestation ratio (van Schaik, 2000b), a variable thought to influence infanticide risk, and hence might relate to female incentives for paternity confusion and mate choice. In such a case, the effect of ecological variation on cycle length should obviously be minimized; however selecting which individual values to include may be difficult, since anomalous variation in itself might be important, and "censored data" will bias estimates of variance in either direction (van Schaik et al., 2000).

Once again, the establishment of a shared database might alleviate some of these complications. Because increasing the explanatory power of comparative analysis is one of the major objectives of obtaining data on new species, access to the raw values used to calculate published means will help investigators make appropriate decisions on the types of data to include in their analyses.

PHYTOSTEROID RESEARCH

One of the major findings of this study was that females experienced seasonal periods of elevated progestins related to *Vitex* availability, and that these periods were associated with both extended cycle lengths and an improved probability of conception. Although we could not rule out nutritional condition as an additional factor explaining patterns of conception, our results were consistent with previous studies in the wild and in captivity, which suggest that the consumption of plant constituents can interfere with endogenous steroids (Higham et al., 2007; Thompson et al., 2008), and that these effects can have negative (Higham et al., 2007; Trisomboon et al., 2006a; Trisomboon et al., 2004; 2005) and/or positive (Milewicz et al., 1993; Westphal et al., 2004) consequences for female fertility.

Overall, these studies suggest that there are additional environmental factors influencing female reproduction that have not been previously addressed. However, there still remain many unanswered questions. For instance, because hormone levels in wild populations have been measured by excreted steroids, particularly in feces, it is unclear to what extent excreted levels reflect: (1) phytosteroids in the plants themselves, (2) absorption of the phytosteroids in circulation, or (3) levels of endogenous hormone whose production, and thus levels, have been affected upstream via phytosteroid interference. Since captive studies show that the doubling the consumption of soy, for example, does not result in a linear increase in the amount of phytoestrogens in the blood stream (Setchell et al., 2003), studying the level of absorption and identifying circulating levels of phytosteroids themselves is crucial. Furthermore, endogenous steroids and phytosteroids can have different binding capacities for the same receptor (Benie and Thieulant, 2003), and varying levels of either might produce different effects on reproduction (e.g., Trisomboon et al., 2004; reviewed in Whitten and Naftolin, 1998; Whitten and Patisaul, 2001). Therefore, distinguishing the levels and function of mammalian steroids from their phytosteroid counterparts is also important for determining the precise cause of reproductive effects.

Most of these problems cannot be addressed on wild populations or with fecal hormone sampling alone. Instead we must rely on experimental studies investigating the relationships between (a) plant consumption, (b) circulating steroid levels, (c) excreted

steroid levels, and (d) reproductive effects. In addition, captive studies that address phytosteroids and reproductive effects in individuals of *varying nutritional condition* might help us to distinguish whether these two important dietary variables have separate or additive reproductive consequences. These studies will not only help improve our understanding of the precise physiological pathways linking phytosteroids to reproductive function, but our ability to interpret patterns found in the wild.

FACTORS INFLUENCING FEMALE & MALE SEXUAL STRATEGIES

A large component of our study addressed patterns of female sexual behavior in relation to paternity confusion and mate choice. We found that overall, the timing of ovulation was not completely concealed, a result consistent with the hypothesis that females might benefit by providing some information about ovulation so that they attract higher quality males during periods of peak fertility (van Schaik et al., 2000). Furthermore, rates of proceptivity and receptivity suggest that females preferred the adult male during fertile periods, but the subadult male during post-conceptive non-fertile periods. Although the preference for the subadult during post-conceptive matings did not characterize non-fertile receptive matings, we interpreted these results to be due, in part, to lack of female interest when the subadult male was younger (when most females were cycling).

Overall, these results support a strategy that balances attraction of high quality males on the one hand and paternity confusion directed towards lower quality males on the other hand (Stumpf and Boesch, 2005). Given that infanticide has never been documented and is generally unlikely in the study population (see chapter 4 for more details), we concluded that paternity confusion might be a successful strategy that females have employed to decrease the risk of infanticide. Additionally, females might offer some probably of paternity in exchange for infant care (Taub, 1980), or (2) support during conflicts with other females.

An additional possibility that we did not discuss is that female non-fertile receptivity might have nothing to do with manipulating males at all. Instead, it has been hypothesized that females exhibit extended receptivity to deplete male sperm, and hence,

delay or inhibit the ability of other females to conceive (Small, 1988). By lowering the conception rates of others, females who engage in non-fertile copulations are thought to increase their own relative fitness. This "sperm depletion" hypothesis has been supported by a few studies showing that female receptive periods exhibit high overlap (Zinner et al., 1994), and that females harass the matings of others (Li and Zhao, 2007; Sommer, 1989). Furthermore, some studies have shown low-ranking females experience a greater number of cycles to conception or delayed pregnancies (Dunbar, 1980; Small, 1988) compared to high-ranking females. However, whether the effect of rank on the probability of conception is truly due to mate competition is open to interpretation (see discussion to follow; reviewed in Soltis, 2002; van Noordwijk and van Schaik, 2000).

Females are expected to engage in mate competition when males are limited, specifically in species/groups characterized by a high female to male ratio (Sommer, 1989; Sommer et al., 1992). With 10 adult females, one adult male, and one breeding subadult male, it could be argued that Phayres's leaf monkeys fall into this category. However, we think the sperm depletion hypothesis is an unlikely explanation for the specific mating patterns found in the present study for several reasons.

First, mating harassments were rare in the study (N = 8), with only one involving an adult female as the harasser. Second, with seven females mating, receptive periods were so common that it was unlikely that females specifically synchronized receptivity (or at least it cannot be statistically tested). Third, there was no obvious relationship with numbers of cycles to conception and average female dominance rank in Phase II of the study (Fig. 5.1; Spearman's rho; $r = 0.075$, $p = 0.873$). And finally, from a theoretical level, even if we did find an effect of female dominance rank on the number of cycles to conception, these effects are just as likely or more likely to be explained by contest competition and differences in female nutrition (reviewed in Soltis, 2002; van Noordwijk and van Schaik, 2000). Therefore, we think the most likely explanations for extended receptivity in Phayre's leaf monkeys remain: (1) a counter-strategy to infanticide, or (2) barter for infant care or male intervention. Future studies should therefore evaluate these hypotheses by collecting additional data on demography (additional years), as well as data on male infant care and male-female relationships in relation to female mating patterns and mate choice, particularly in additional multi-male groups.

MALE KNOWLEDGE

Evaluating the degree to which different males have knowledge of fertility might be an additional avenue for future research. In this study, we found that the subadult male was more often involved in post-conceptive receptive periods relative to the adult male. Despite this difference, adult males rarely harassed matings. This finding is consistent with several other studies on primates (Carnegie et al., 2005; Engelhardt et al., 2007; Gust, 1994) and suggests that male variation in the ability to discriminate female fertility might be an important factor influencing both male and female strategies.

Engelhardt et al. (2007) have suggested that because males with most frequent access to females during fertile periods (e.g., dominant vs. subordinate males, resident vs. non-resident males) seem the least interested in females during gestation, the ability to discriminate broad-scale differences in reproductive status might be experience-based. In other words, male discriminating ability might be influenced by the amount of time they are exposed to potential cues. If close exposure is required, the mechanism of communication is likely via olfaction. Primate males often inspect females by smell (e.g., Clarke et al., 2009), and it is possible that frequent inspections result in an ability to recognize fertile versus infertile periods (Higham et al., 2009). Gust (1994) has suggested that, at least for post-conceptive mating, this olfactory cue might be based on the recognition of luteinizing hormone (LH). Although estrogen and progesterone are both secreted at fairly high levels around ovulation and during gestation, LH is not. This is because LH stimulates the ovary to secrete estrogen, but the production of estrogen is taken over by the corpus luteum during early pregnancy, and the placenta roughly five to six weeks into gestation (based on data from rhesus macaques: Atkinson et al., 1975). Hence, during gestation, the olfactory signal of luteinizing hormone should be absent (Gust, 1994). Future experimental studies that manipulate (1) female hormone levels (via implants: e.g., Glick et al., 1982) and (2) female vaginal scents (e.g., Cerda-Molina et al., 2006) should clarify whether such a hormonal cue exists, whether signals have an olfactory basis, and whether exposure indeed improves an individual's ability to discriminate the cue. If experience does emerge as a significant factor, studies in the wild

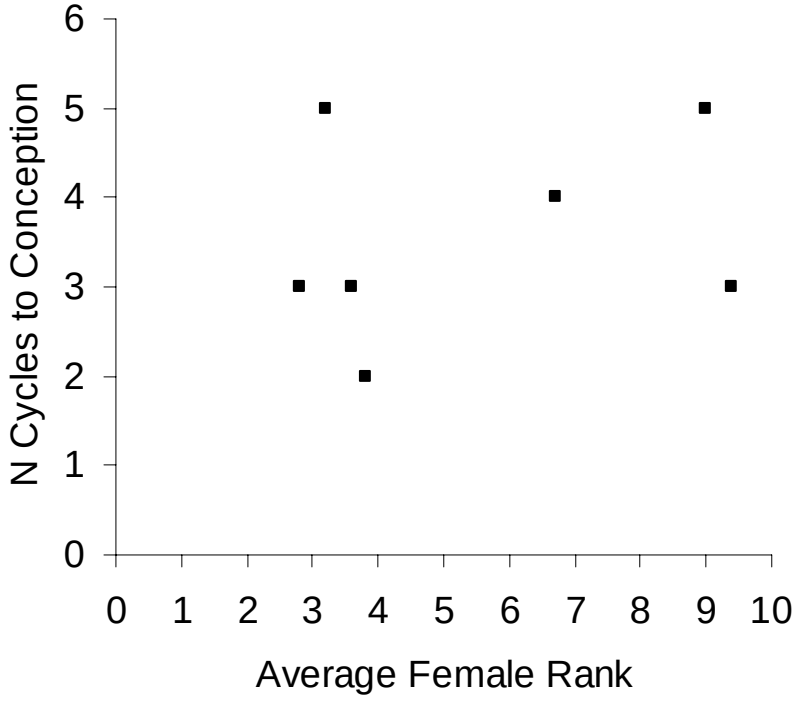
would expect the ability of adult males and non-resident males to both vary with exposure; however, nearly all subadult males would be poor discriminators.

Results from such studies would have broad ramifications for interpreting male and female sexual strategies. For instance, if all males discriminate fertility equally, we must conclude that subordinate males mate with females during non-fertile periods with full knowledge that the probability of conception is extremely low. Thus males might be mating with females for non-procreative benefits (Carnegie et al., 2005). At the same time, if post-conceptive mating does not succeed in manipulating paternity assessements, extended post-conceptive receptivity may not result in the expected benefits of paternal care, decreased infanticide risk, or increased infanticide protection. Studies on Hanuman langurs already suggest that this might be the case. Specifically, males in this population were less likely to commit infanticide if they mated with cycling females, but not with pregnant females (Borries et al., 1999). On the other hand, if males indeed show variability in discrimination, that variability plays perfectly into the dual strategy hypothesized for females: to mate with high quality males while at the same time deceiving the rest (van Schaik, 2000b).

Table 5.1 Comparative data on cycle length, and the lengths of the follicular and luteal phase in colobine species. (C = Captive, W = Wild, H = Hormone-based, M = Menstruation-based). Results for the present study from top to bottom listed as species typical values (normal progestin period without B12), overall values, values for high progestin only, values for low progestin period (complete data set), values for high progestin without considering B12. Data from Gibson and Chan (1992) and Ruempler (1998) were not included because clear methods for assessing cycle length, as well as sample sizes and means, were not provided.

				Cycle (days)		Follicular (days)		Luteal (days)		
Species	**C/W**	**Method**	**N**	**mean**	**SEM**	**mean**	**SEM**	**mean**	**SEM**	**Source**
Colobus guereza	W	H	6	22.4	1.6	NA	NA	NA	NA	Harris & Monfort, 2006
Pygathrix nemaeus	C	H	5-9	26.4	0.8	13.1	1.0	14.8	0.5	Heistermann et al., 2004
Rhinopithecus bieti	C	H	3	23.6	2.0	13.2	1.0	11.5	0.7	He et al., 2001
	C	M	10	26.1	0.5					Weizhi et al., 1998
Semnopithecus entellus	C	H	7	25.0	2.1	12.1	2.1	12.9	0.3	Heistermann et al., 1995
	W	H	6	27.0	1.9	12.8	1.5	14.2	1.1	Ziegler, 2001; Heistermann et al., 2001
Trachypithecus phayrei crepusculus	W	H	**16-17**	**28.4**	**1.6**	**15.4**	**1.3**	**12.4**	**0.4**	**Present study (species typical value)**
			18	38.7	4.1	25.7	3.9	12.2	0.5	Present study (complete dataset)
			18	50.6	7.8	37.9	7.2	11.3	1.7	Present study (high progestin, complete dataset)
			18	31.2	3.1	18.0	2.9	12.5	0.4	Present study (normal progestin, complete dataset)
			16-17	44.0	5.0	31.0	3.8	11.3	1.7	Present sudy (high progestin –B12)

Figure 5.1 Average female ranks across nine months (Jan-Sep, 2006) during Phase II of the study in relation to number of cycles to conception. No significant relationships between the two (Spearman's rho; r = 0.075, p = 0.873).



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APPENDIX I

SUMMARY OF LABORATORY PROTOCOLS

Detailed protocols for (1) fecal extraction, and (2) radioimmunoassay (RIA) for the measurement of fecal (a) estrogens and (b) progestins are described in the following pages. For RIA protocols, the specific schedule and volumes of adding reagents are detailed in subsequent tables (Table A1.1, A1.2).

FECAL EXTRACTION PROTOCOL

Description

Ethanol-based fecal extraction protocol following methods developed by Wasser et al. (1994).

Reagents

100% Ethanol
Distilled water

Extraction Steps

1. Lyophilize wet samples.
2. Sift dried samples through fine mesh wire.
3. Weigh out 0.1 g dry powdered feces to plastic tubes (8mm o-ring cap).
4. Add 500 µl distilled water and 500 µl EtOH to each tube and vortex very briefly.
5. Add 4.0 ml of EtOH to each tube and vortex briefly.
6. Boil tubes in boiling water bath (85-90ºC degrees) for 20 minutes.
7. Centrifuge at 1500 rpm for 20 minutes.
8. Decant supernatant into glass tubes (16x100 mm).
9. To the first tube containing the fecal pellet, add 4.5 mls EtOH and 500 µl of distilled water and vortex for 30 seconds.
10. Centrifuge at 1500 rpm for 15 minutes.
11. Decant supernatant into the second (16x100 mm glass) tube.
12. Dry out samples in 37° C water under nitrogen gas with an Evaporac.
13. When samples are dry, resolubilize fecal samples in 1 ml of EtOH.
14. Vortex.
15. Decant into 2 ml microtubes.
16. Store in -20ºC freezer until further use.

RIA PROTOCOL FOR FECAL ESTROGENS

Description

Assay protocols modified (by A.L.) from protocols provided by MP Biomedicals for direct E2 double antibody RIA. Reagents for all kit components were halved for assay.

Reagents (all but controls provided with kit)

1. Diluent buffer (MP Biomedicals, Solon, OH)
2. 1^{st} E2 antibody (MP Biomedicals, Solon, OH)
3. ^{125}I – labeled E2 tracer (MP Biomedicals, Solon, OH)
4. Precipitating solution (2^{nd} AB) (MP Biomedicals, Solon, OH)
5. E2 standards (MP Biomedicals, Solon, OH)
6. Controls (lyphocheck III, BioRad, Hercules, CA)
7. Pooled sample controls

Preparatory Steps

1. Make lyphocheck III control dilutions (low: 1:10; mid: 1:6, high: 1:1).
2. Make pooled sample dilutions (low: 1:500; mid: 1:100, high: 1:20).

Assay Steps (Table A1-1 for schedule & exact amounts)

1. Label tubes 1-200.
2. Remove samples from the freezer and let thaw.
3. Remove reagents from the refrigerator.
4. Pipette samples in appropriate dilution (50 µl of 1:8 for non-pregnant, 10-100 µl of 1:100 for pregnant) in tubes 31-100.
5. Pipette 50 µl pooled sample controls (low: 1:500; mid: 1:100, high: 1:20).
6. Dry down samples and pooled sample controls under nitrogen gas with an Evaporac.
7. Pipette 25 µl of standards (1-7) in tubes 5-18, and diluted lypocheck III controls (low: 1:10; mid: 1:6, high: 1:1) in tubes 18-24 (Table A1-1).
8. Add diluent buffer, standard 1, primary antibody, and tracer in appropriate sequence and amounts (Table A1-1).
9. Vortex and incubate at 37ºC for 90 minutes.
10. Add precipitating solution (Table A-1-1).
11. Centrifuge at 4ºC for 20 minutes at 2500 rpms.
12. Aspirate and count in the gamma counter.

RIA PROTOCOL FOR FECAL PROGESTINS

Description

RIA using CL 425 as 1st AB (C. Munroe, UC Davis, CA), Goat Anti-Mouse IgG as 2nd AB (L. Sibley, UC Davis, CA), and Pantex Standards & Reagents. Protocol developed by Laurence Gesquiere, Princeton University.

Reagents

1. RIA Buffer (PBS/BSA/Tween Buffer)
2. PEG Buffer (5% PEG in PBS Buffer)
3. 1st P4 AB (1:12000 dilution, CL 425, C. Munroe, Davis, CA)
4. 2nd AB (Goat-Anti-Mouse IgG, Equitech-Bio, Kerrville, TX)
5. Mouse serum (Sigma Aldrich, St. Louis, MO)
6. Progesterone Standards (Pantex, Santa Monica, CA)
7. Progesterone ^{125}I - labeledTracer (Pantex, Santa Monica, CA)
8. Lypocheck II (BioRad, Hercules, CA)

Preparatory Steps

I) Buffer preparation

1. RIA buffer**:** PBS/BSA/Tween buffer (1 liter):
   8g NaCl
   0.2g KCl
   1.44g Na_2HPO_4
   0.24g NaH_2PO_4
   - Stir at low heat (Graduation 2 for heat on plate)
   - Once dissolved add:
     1g BSA (Bovine Serum Albumin)
     1g Tween 80
   - After complete dissolution, adjust pH to 7.5 (add NaOH if pH lower than 7.5)

2. PEG buffer: 5% PEG in PBS buffer (1 liter):
   8g NaCl
   0.2g KCl
   1.44g Na_2HPO_4
   0.24g NaH_2PO_4
   - Stir at low heat (Graduation 2 for heat on plate)
   - Once dissolved add:
     50 g PEG 8000 (Polyethylene Glycol)
   - After complete dissolution, adjust pH to 7.5 (add NaOH if pH lower than 7.5)

II) Antibody and serum preparation

1. 1st AB (CL425) d1/12000
   40µl 1st AB d1/10
   48 ml PBS/BSA/Tween Buffer
2. Normal mouse serum 1% (Sigma)
   500µl serum
   49.5ml PEG Buffer
3. 2nd AB: Goat-Anti-Mouse IgG (Equitech-Bio GAMG-0100) d1/100:
   10 ml 2nd AB
   1000 ml PEG Buffer

   if only for 1 RIA:
   2 ml 2nd AB
   200 ml PEG Buffer

III) Control Preparation

3. Lyphocheck III control dilutions (low: 1:32; mid: 1:8, high: 1:2).
4. Pooled sample dilutions (low: 1:1000; mid: 1:250, high: 1:60).

Assay Steps (Table A1-2 for schedule & exact amounts)

13. Label tubes 1-200.
14. Remove samples from the freezer and let thaw.
15. Remove reagents from the refrigerator.
16. Pipette samples in appropriate amount and dilution (50 µl of 1:16 for non-pregnant, 10-200 µl of 1:200 for pregnant) in tubes 35-200.
17. Pipette 50 µl pooled sample controls (low: 1:1000; mid: 1:250, high: 1:60) in tubes 29-34.
18. Dry samples and pooled sample controls under nitrogen gas with an Evaporac.
19. Pipette 25 µl standards (1-9) in tubes 5-22, and diluted lyphocheck controls (low: 1:32; mid: 1:8, high: 1:2) in tubes 23-28 (Table A1-2).
20. Add RIA Buffer, standard 1, primary antibody, and tracer in the appropriate sequence and amounts (Table A1-2).
21. Vortex and incubate at room temperature over-night.
22. Add mouse serum and 2nd AB in appropriate amounts (Table A1-2).
23. Vortex and incubate at room temperature for 1 hour.
24. Centrifuge at room temperature 20 minutes at 3100 rpms.
25. Aspirate and count in the gamma counter.

Table A1-1 Schedule of adding samples and reagents in estrogen RIA.

MP Biomedicals Estradiol (E2) RIA	Tube #	Sdt 1 or con (µl)	Diluent Buffer (µl)	1st AB (µl)	E2 Tracer (µl)	Vortex, incubate at 37°C for 90 min	Prec 2nd AB (µl)	Centrifuge at 4°C for 20min at 2500 rpm Aspirate and count
TC	1-2	-	525	-	250		-	
NSB	3-4	25	250	-	250		250	
B0 (Sdt1)	5-6	25	-	250	250		250	
Sdt 2	7-8	25	-	250	250		250	
Sdt 3	9-10	25	-	250	250		250	
Sdt 4	11-12	25	-	250	250		250	
Sdt 5	13-14	25	-	250	250		250	
Sdt 6	15-16	25	-	250	250		250	
Sdt 7	17-18	25	-	250	250		250	
Lypo III (1:10)	19-20	25	-	250	250		250	
Lypo III (1:6)	21-22	25	-	250	250		250	
Lypo III (1:1)	23-24	25	-	250	250		250	
Pool low (1:500)	25-26	25	-	250	250		250	
Pool mid (1:100)	27-28	25	-	250	250		250	
Pool hi (1:20)	29-30	25	-	250	250		250	
Samples	31-200	25	-	250	250		250	

Table A1-2 Schedule of adding samples and reagents in progestin RIA.

P4 RIA (CL 425)	Tube #	Sdt 1 or con (µl)	RIA Buffer (µl)	1st AB (µl)	Tracer Pantex (µl)	Overnight incubation at room T°C	Mouse serum (µl)	2nd AB (ml)	Incubate for 1hr at room T°C. Centrifuge at room T°C for 20min at 3100 rpm, Aspirate and count
TC	1-2	-	1250	-	200		-	-	
NSB	3-4	50	100	-	200		100	1	
B0 (Sdt1)	5-6	50	-	100	200		100	1	
Sdt 2	7-8	50	-	100	200		100	1	
Sdt 3	9-10	50	-	100	200		100	1	
Sdt 4	11-12	50	-	100	200		100	1	
Sdt 5	13-14	50	-	100	200		100	1	
Sdt 6	15-16	50	-	100	200		100	1	
Sdt 7	17-18	50	-	100	200		100	1	
Sdt 8	19-20	50	-	100	200		100	1	
Sdt 9	21-22	50	-	100	200		100	1	
Lypocheck II (1:32)	23-24	50	-	100	200		100	1	
Lypocheck II (1:8)	25-26	50	-	100	200		100	1	
Lypocheck II (1:2)	27-28	50	-	100	200		100	1	
Pool low	29-30	50	-	100	200		100	1	
Pool mid	31-32	50	-	100	200		100	1	
Pool high	33-34	50	-	100	200		100	1	
Samples	35-200	50	-	100	200		100	1	

APPENDIX II

INDIVIDUAL HORMONE AND BEHAVIOR PROFILES

In this appendix, individual hormone and behavior profiles for 10 of the 11 females in the study group are presented. Figures are arranged by female, with (1) complete hormone profiles for the entire 20-month study period for all 10 females, profiles with details of both hormones and the timing of receptive periods from cycling to conception for seven (B2, B3, B5, B6, B7, B9, B12) of the ten females who were followed extensively during Phase II of the study (2006), and profiles with the same details on hormones and receptive periods for six of these females (B2, B3, B5, B6, B9, B12) during the post-conceptive period of the same year. No post-conceptive profile of hormones and behavior is provided for female B7 because she conceived within a month prior to the end of the study period and did not exhibit any post-conceptive sexual behavior. All profiles with behavioral data include only the time from February 1, 2006 onward, when field assistants were properly trained for the collection of sexual behavior.

Figure A2.1a Hormone profile for female B2 during entire study period (solid line = fE (ng/g feces); dotted line = fP (µg/g feces); dotted arrow = birth date; solid arrow = conception date).

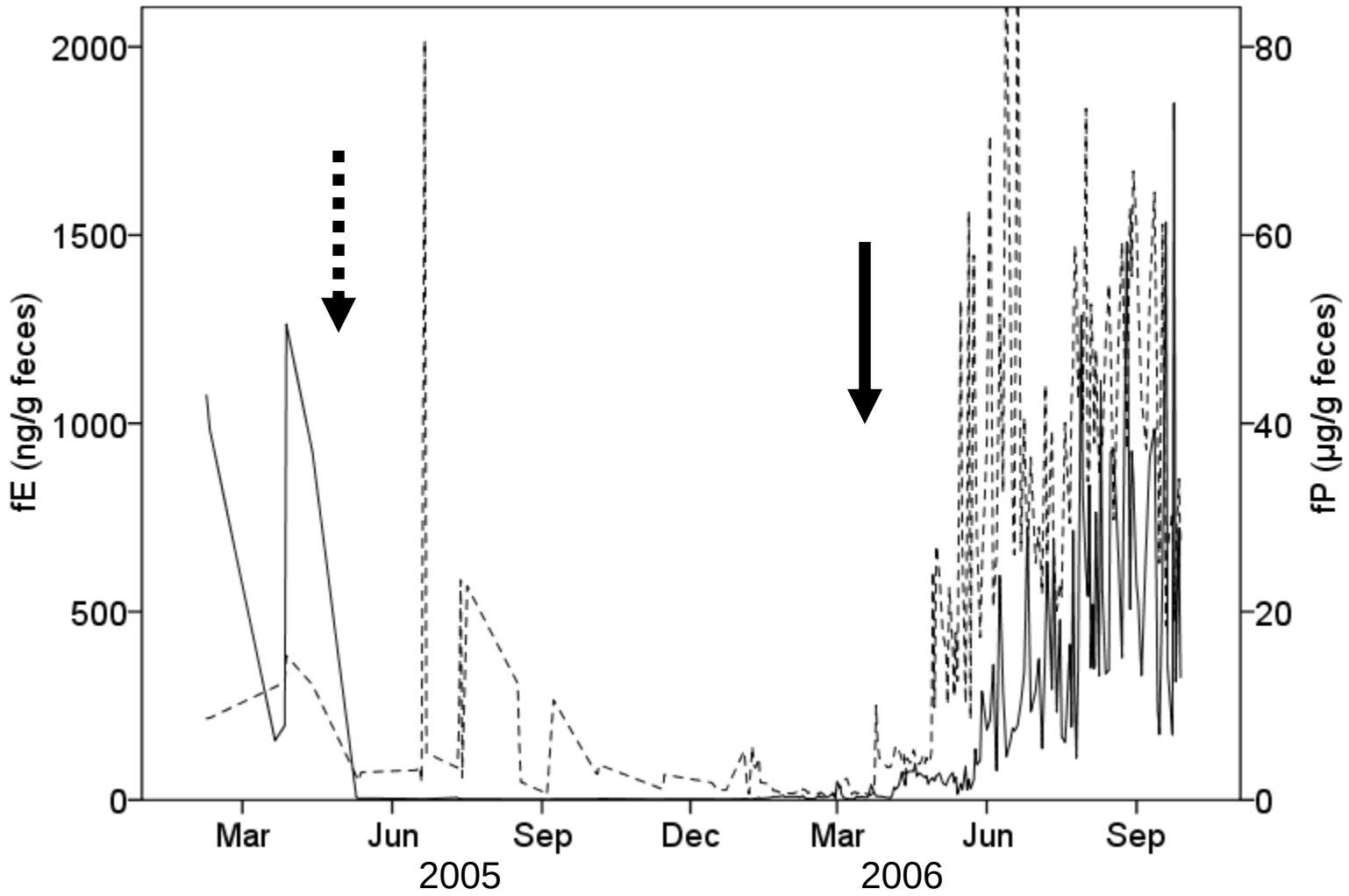

Figure A2.1b Hormone and behavior profile for female B2 prior to conception in 2006 (solid line = fE (ng/g feces); dotted line = fP (µg/g feces); solid arrow = conception date; black horizontal bars = receptive periods).

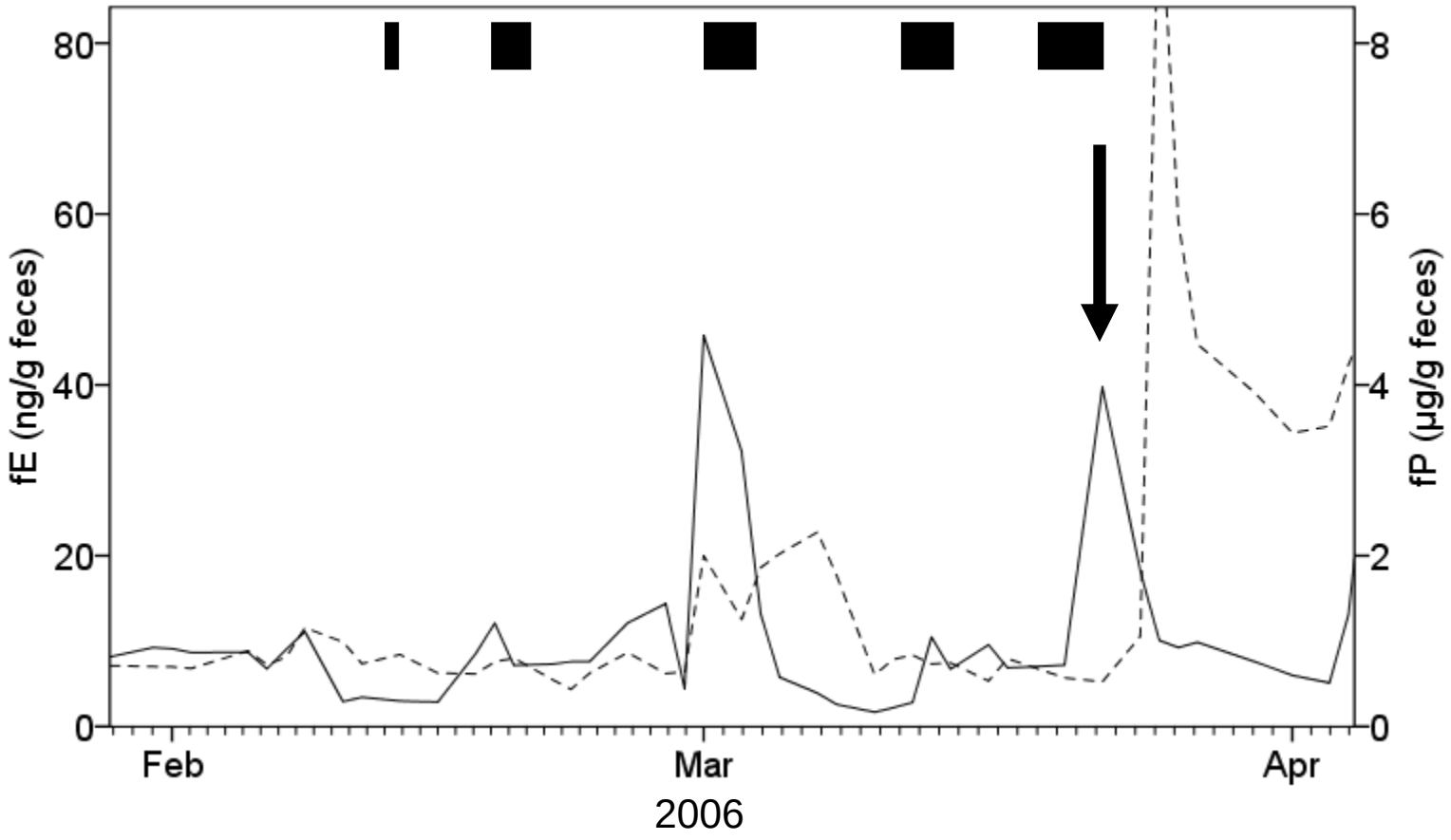

Figure A2.1c Hormone and behavior profile for B2 post-conception in 2006 (solid line = fE (ng/g feces); dotted line = fP (µg/g feces); solid arrow = conception date; black horizontal bars = receptive periods).

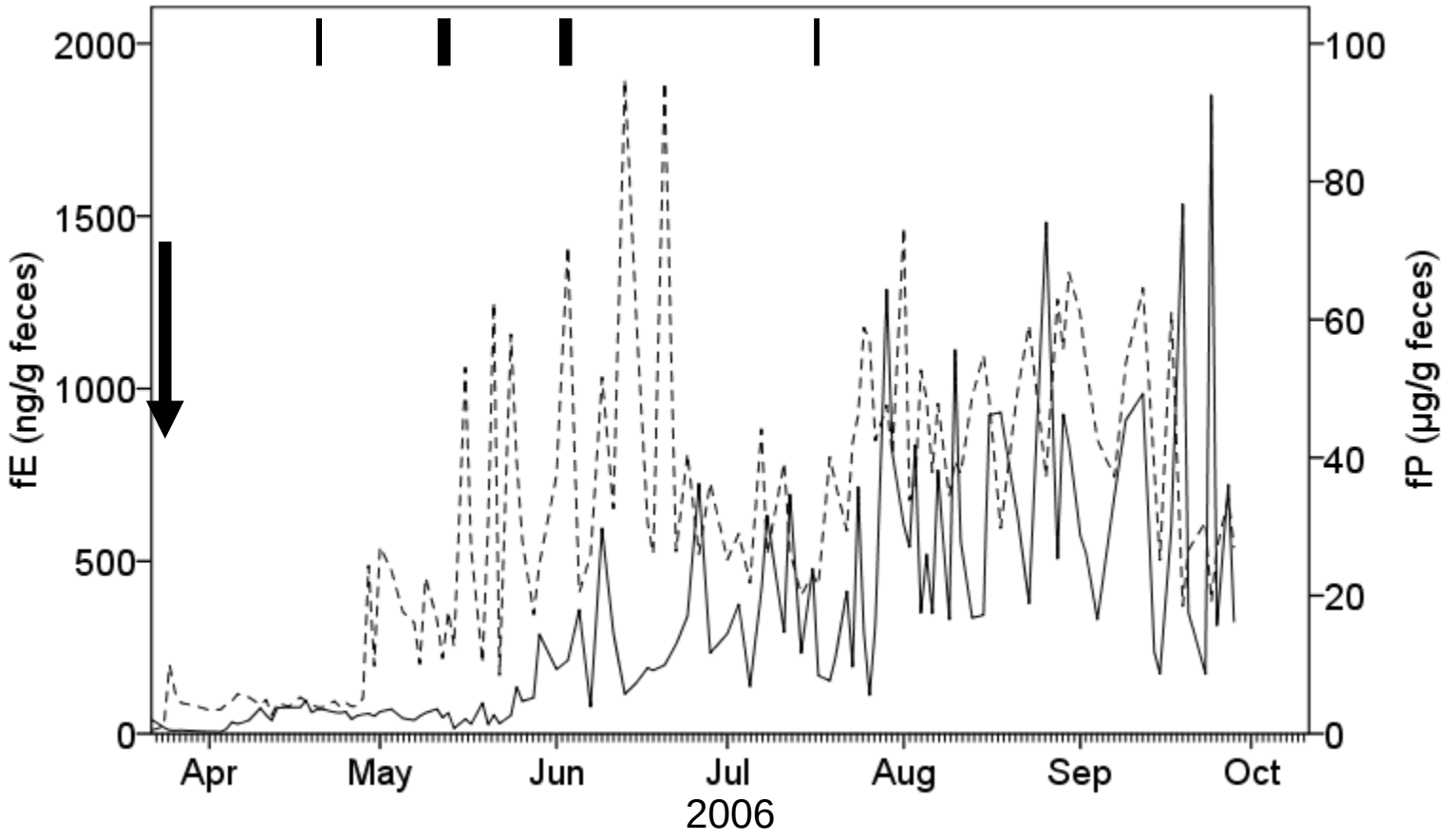

Figure A2.2a Hormone profile for female B3 during entire study period (solid line = fE (ng/g feces); dotted line = fP (µg/g feces); solid arrow = conception date).

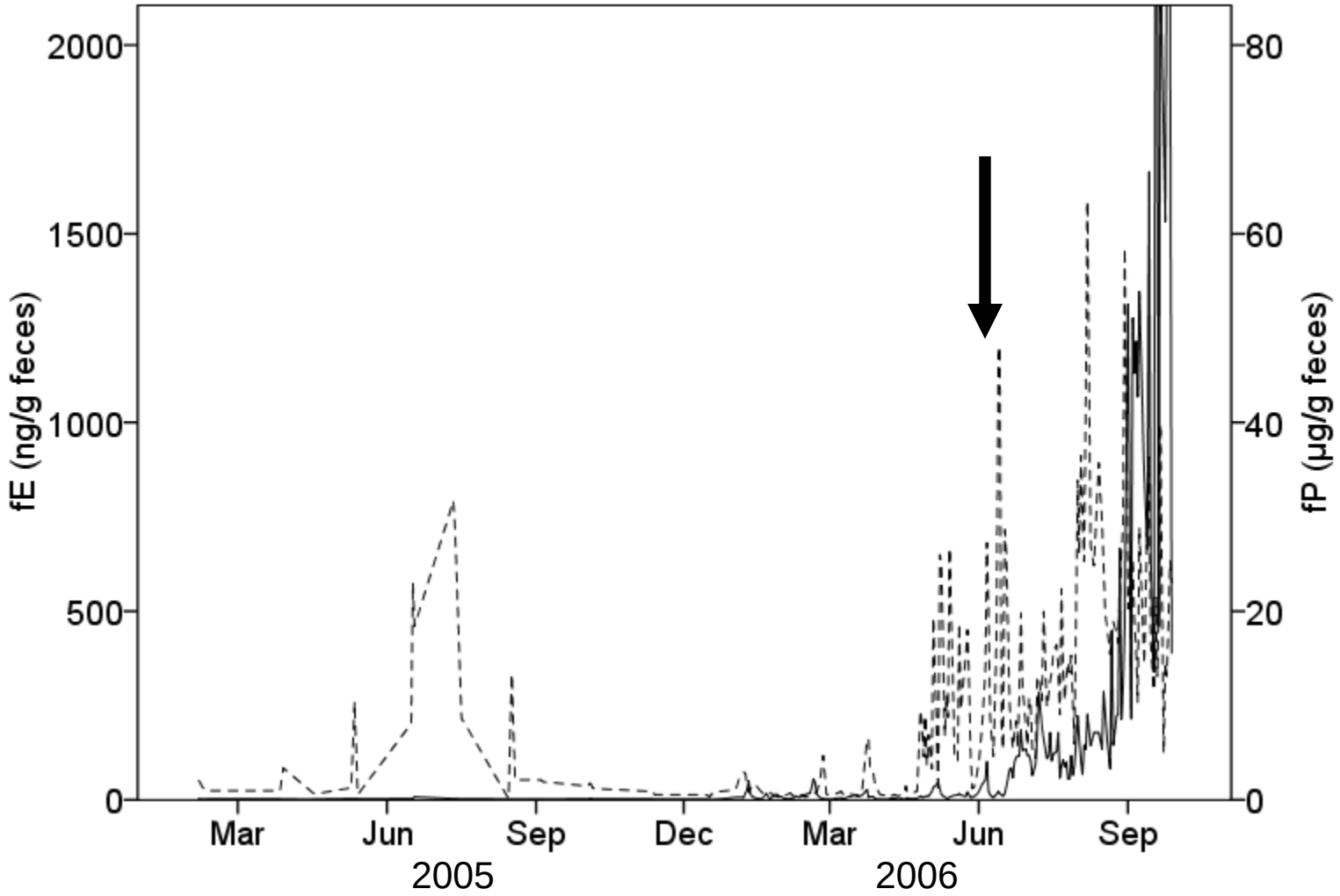

Figure A2.2b Hormone and behavior profile for female B3 prior to conception in 2006 (solid line = fE (ng/g feces); dotted line = fP (μg/g feces); solid arrow = conception date; black horizontal bars = receptive periods).

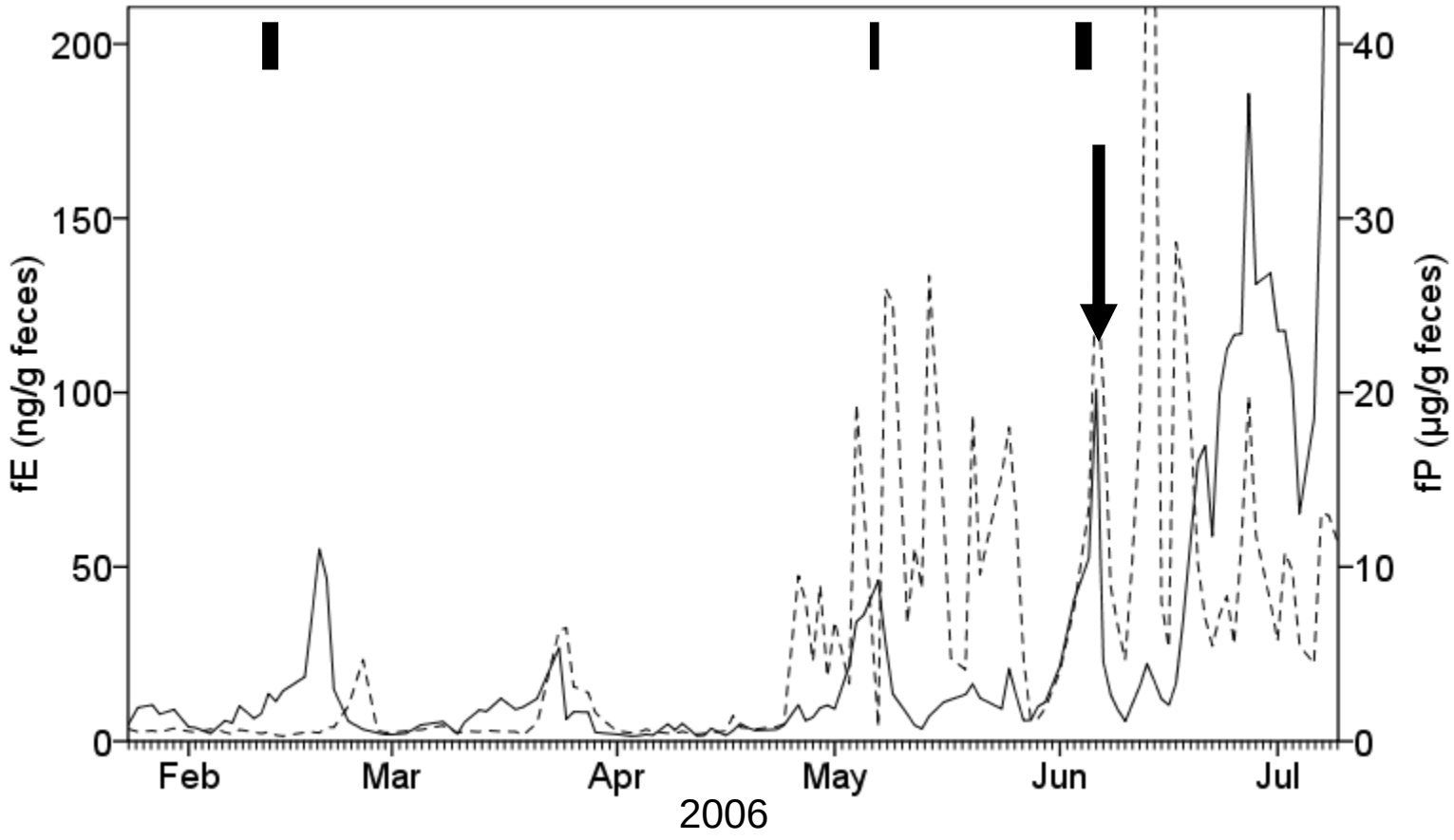

Figure A2.2c Hormone and behavior profile for B3 post-conception in 2006 (solid line = fE (ng/g feces); dotted line = fP (µg/g feces); solid arrow = conception date; black horizontal bars = receptive periods).

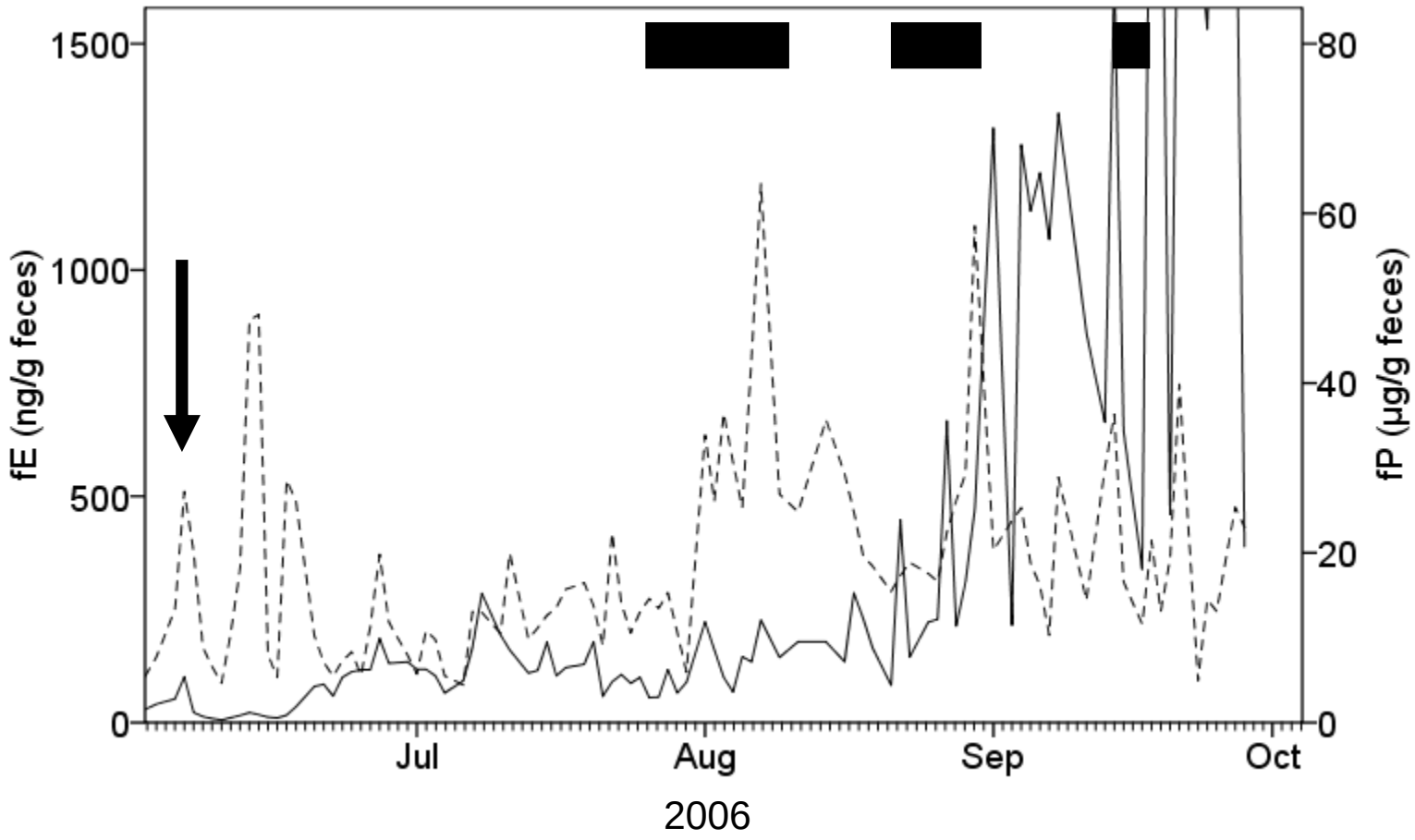

Figure A2.3 Hormone profile for female B4 during entire study period (solid line = fE (ng/g feces); dotted line = fP (µg/g feces); dotted arrow = birth date; solid arrow = conception date).

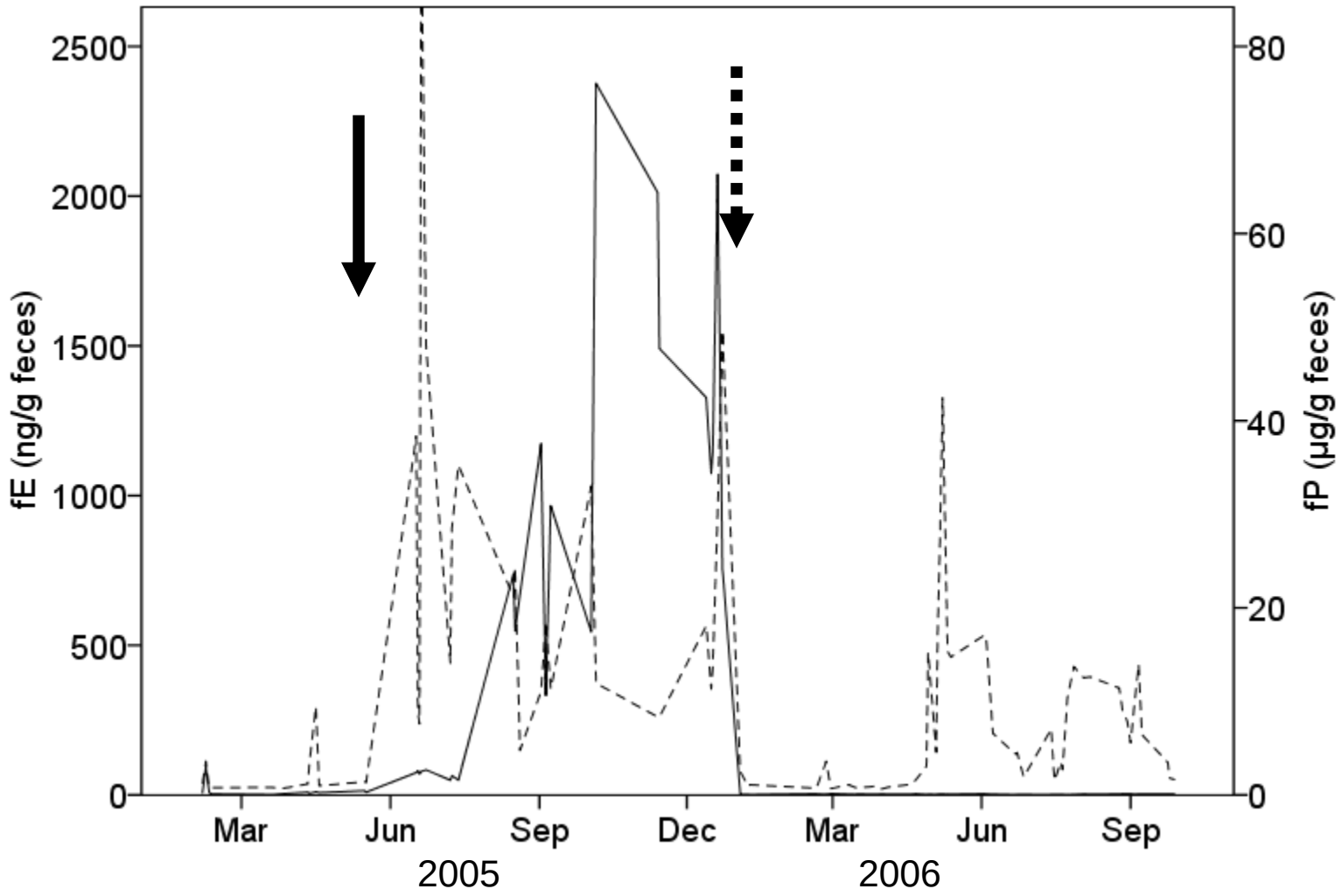

Figure A2.4a Hormone profile for female B5 during entire study period (solid line = fE (ng/g feces); dotted line = fP (µg/g feces); dotted arrow = birth date; solid arrow = conception date).

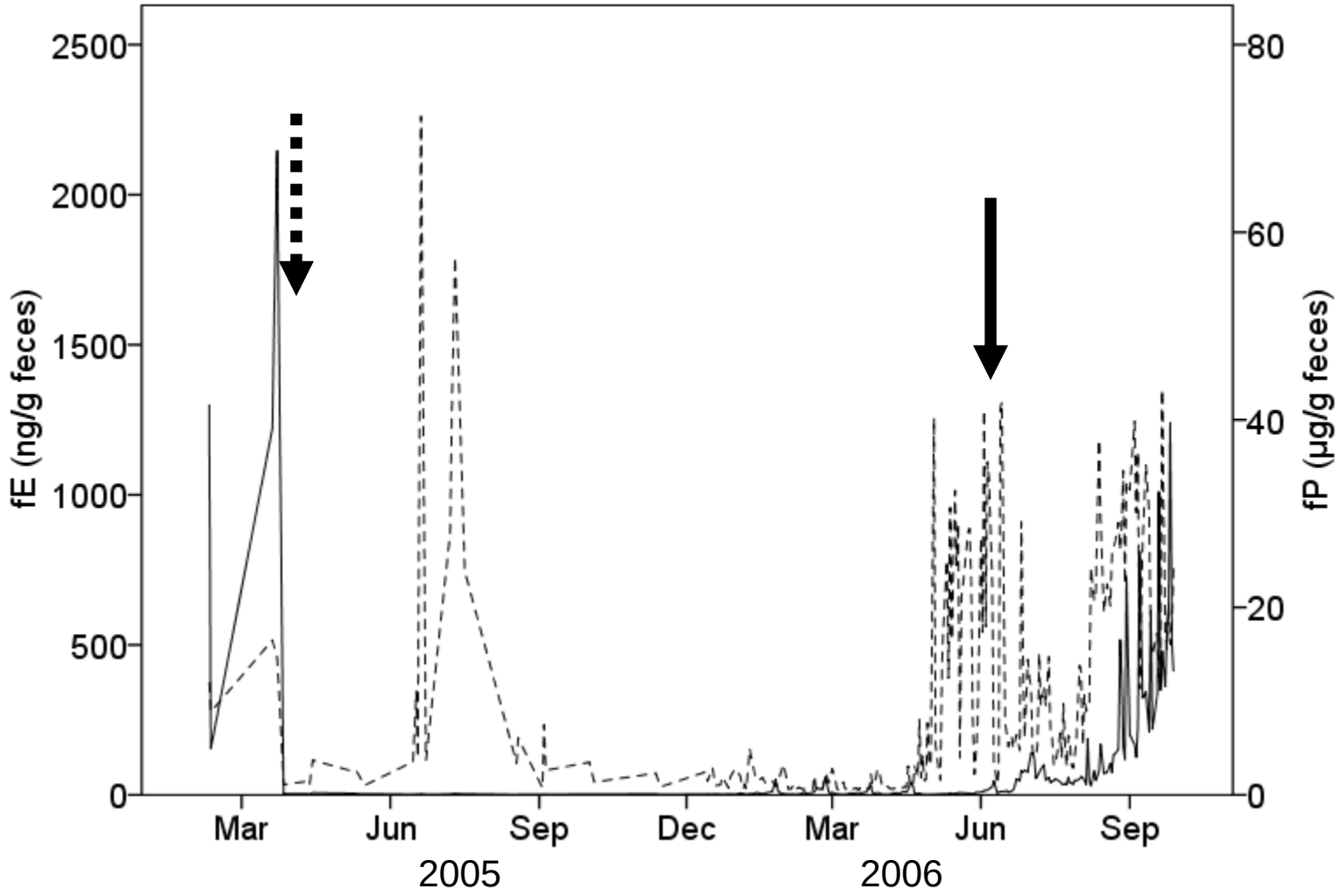

Figure A2.4b Hormone and behavior profile for female B5 prior to conception in 2006 (solid line = fE (ng/g feces); dotted line = fP (µg/g feces); solid arrow = conception date; black horizontal bars = receptive periods).

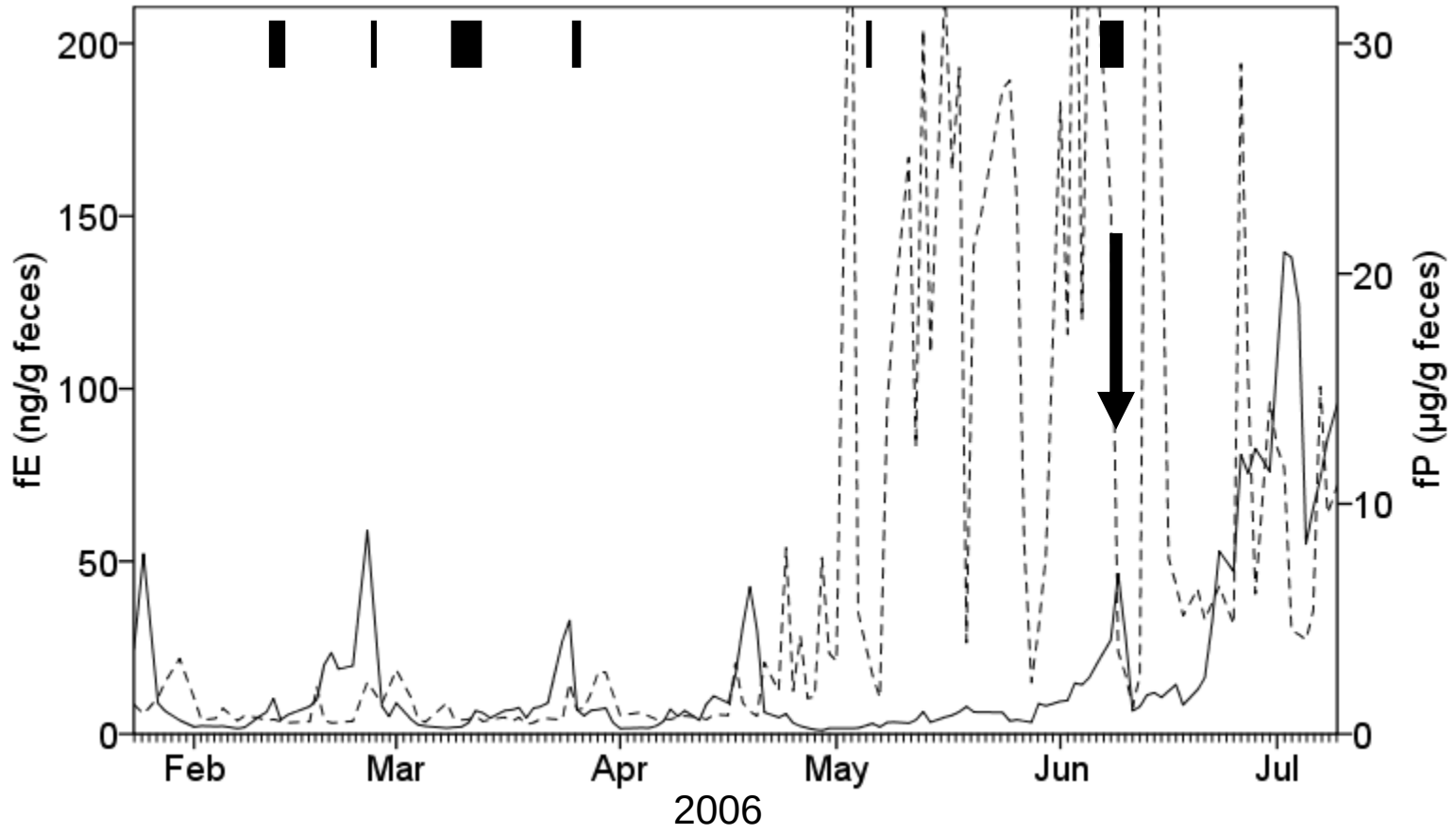

Figure A2.4c Hormone and behavior profile for B5 post-conception in 2006 (solid line = fE (ng/g feces); dotted line = fP (µg/g feces); solid arrow = conception date; black horizontal bars = receptive periods).

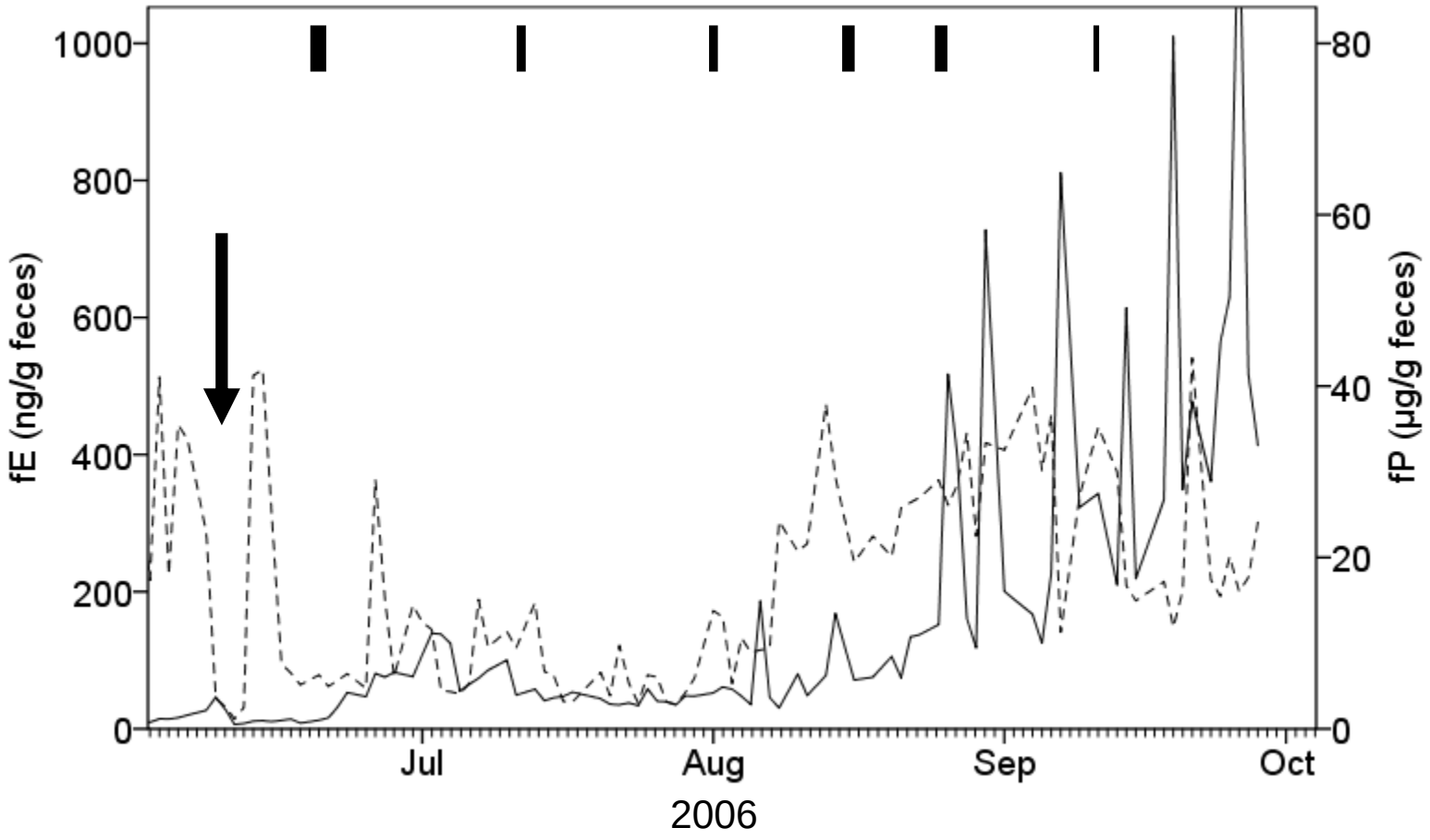

Figure A2.5a Hormone profile for female B6 during entire study period (solid line = fE (ng/g feces); dotted line = fP (µg/g feces); solid arrow = conception date).

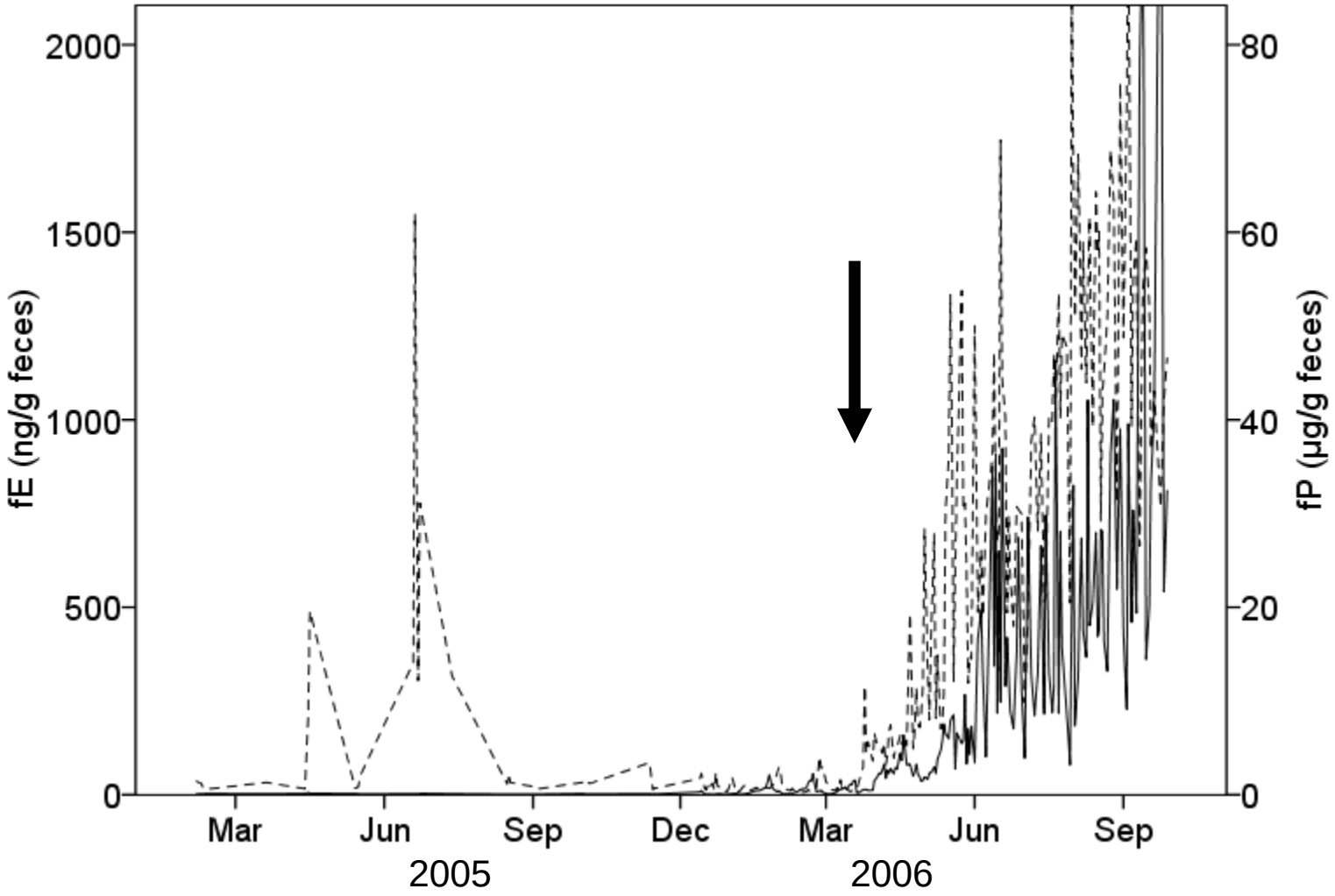

Figure A2.5b Hormone and behavior profile for female B6 prior to conception in 2006 (solid line = fE (ng/g feces); dotted line = fP (µg/g feces); solid arrow = conception date; black horizontal bars = receptive periods).

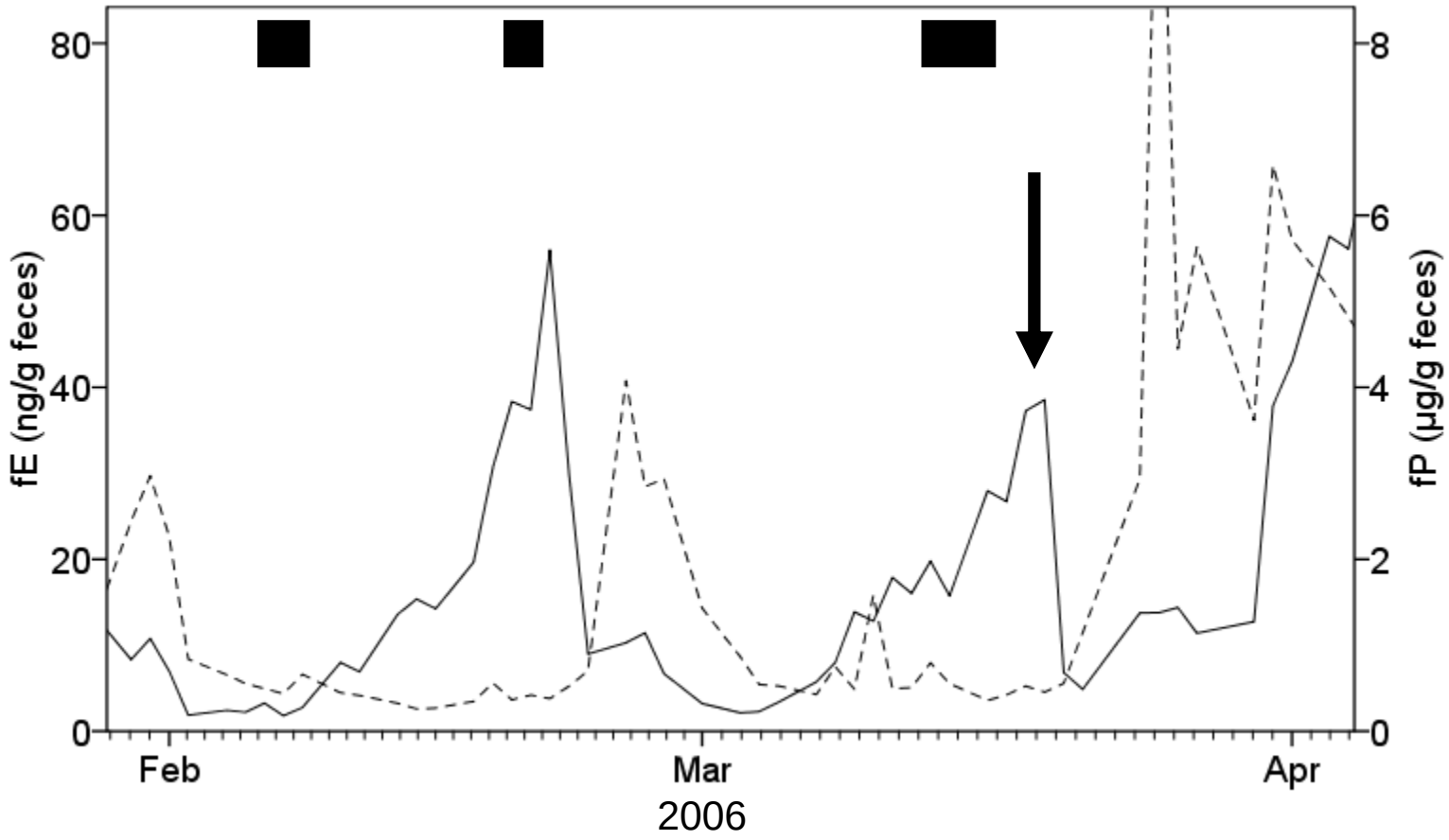

Figure A2.5c Hormone and behavior profile for B6 post-conception in 2006 (solid line = fE (ng/g feces); dotted line = fP (µg/g feces); solid arrow = conception date; black horizontal bars = receptive periods).

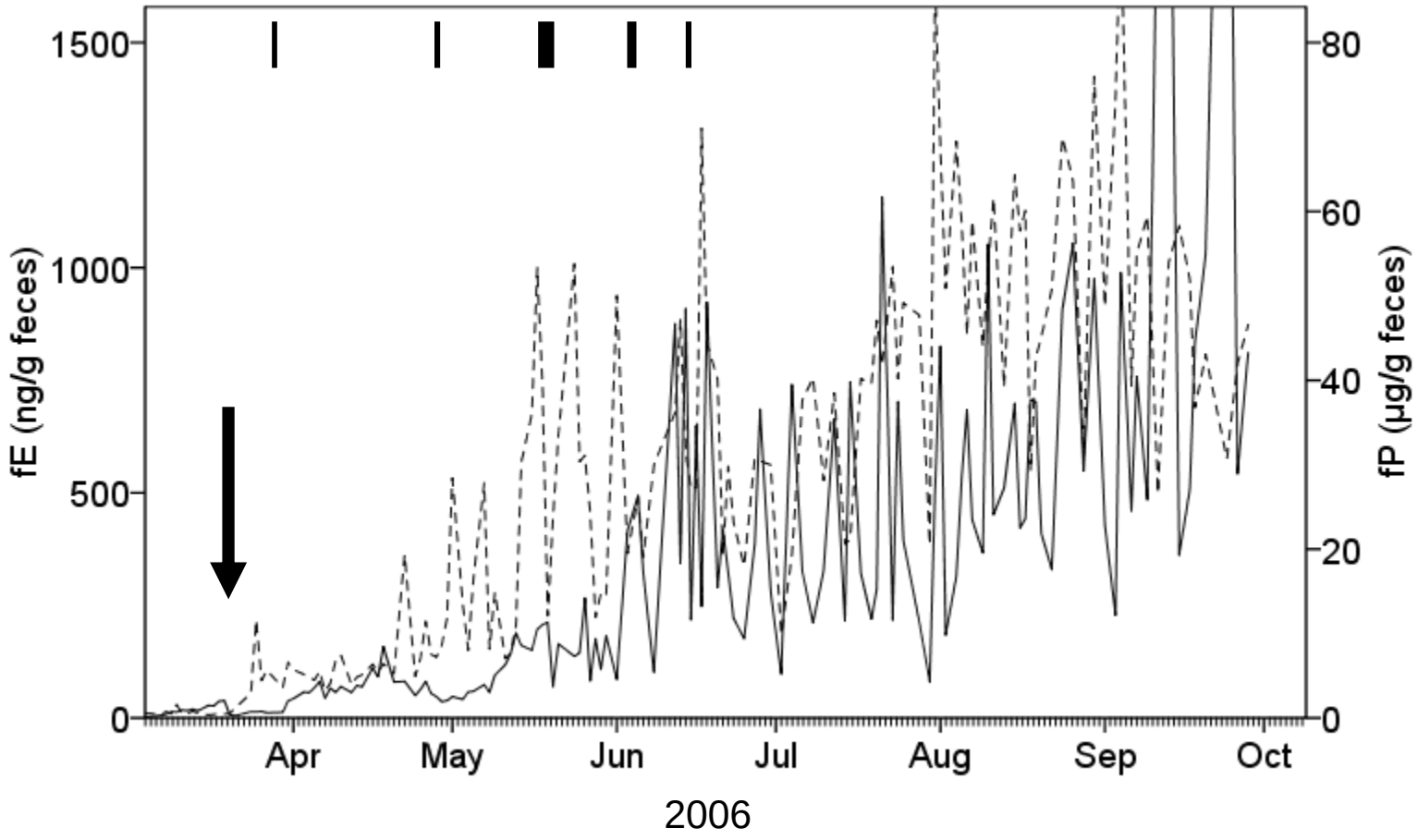

Figure A2.6a Hormone profile for female B7 during entire study period (solid line = fE (ng/g feces); dotted line = fP (µg/g feces); dotted arrow = birth date; solid arrow = conception date).

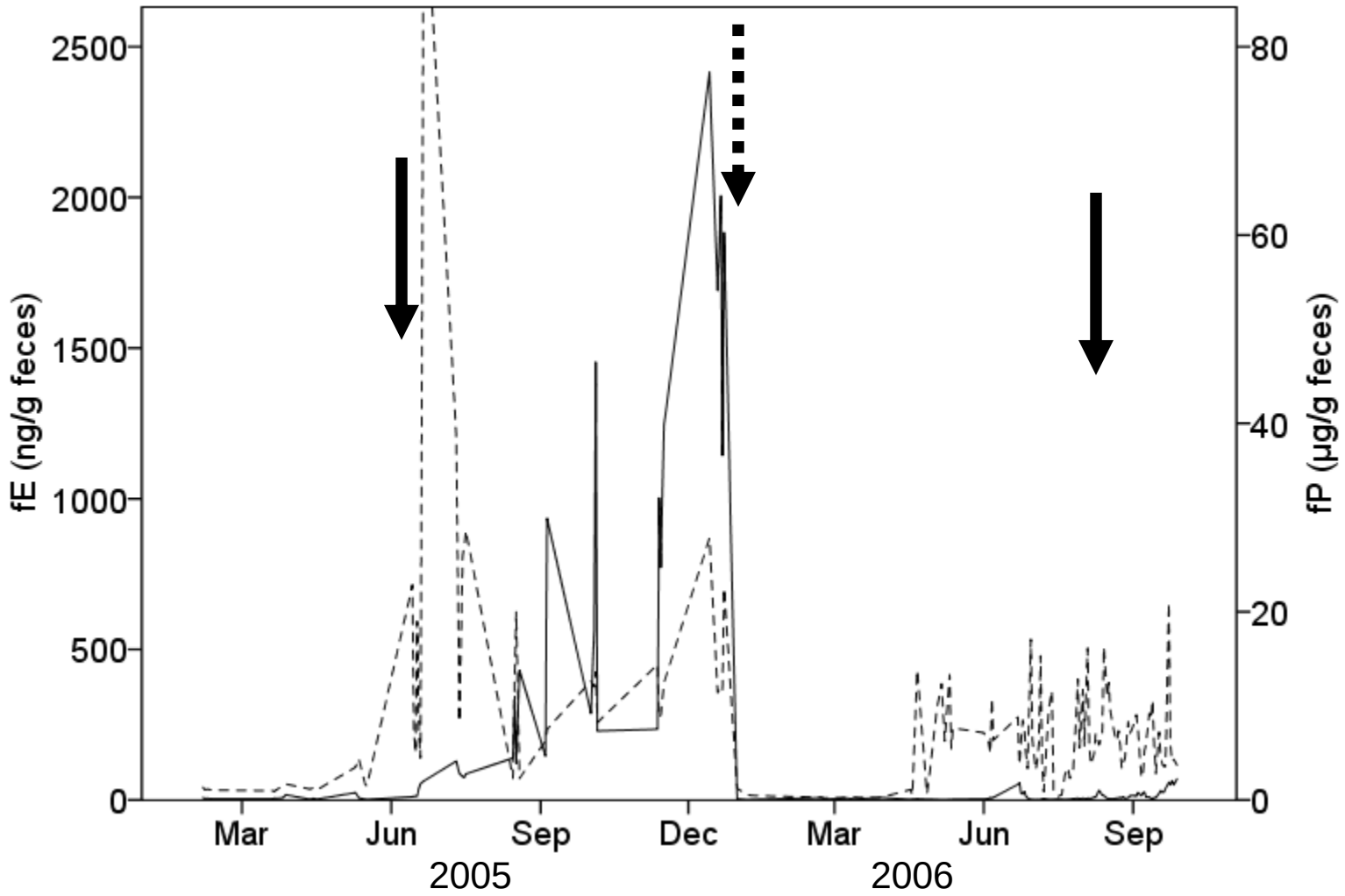

Figure A2.6b Hormone and behavior profile for female B7 prior to conception in 2006 (solid line = fE (ng/g feces); dotted line = fP (μg/g feces); solid arrow = conception date; black horizontal bars = receptive periods).

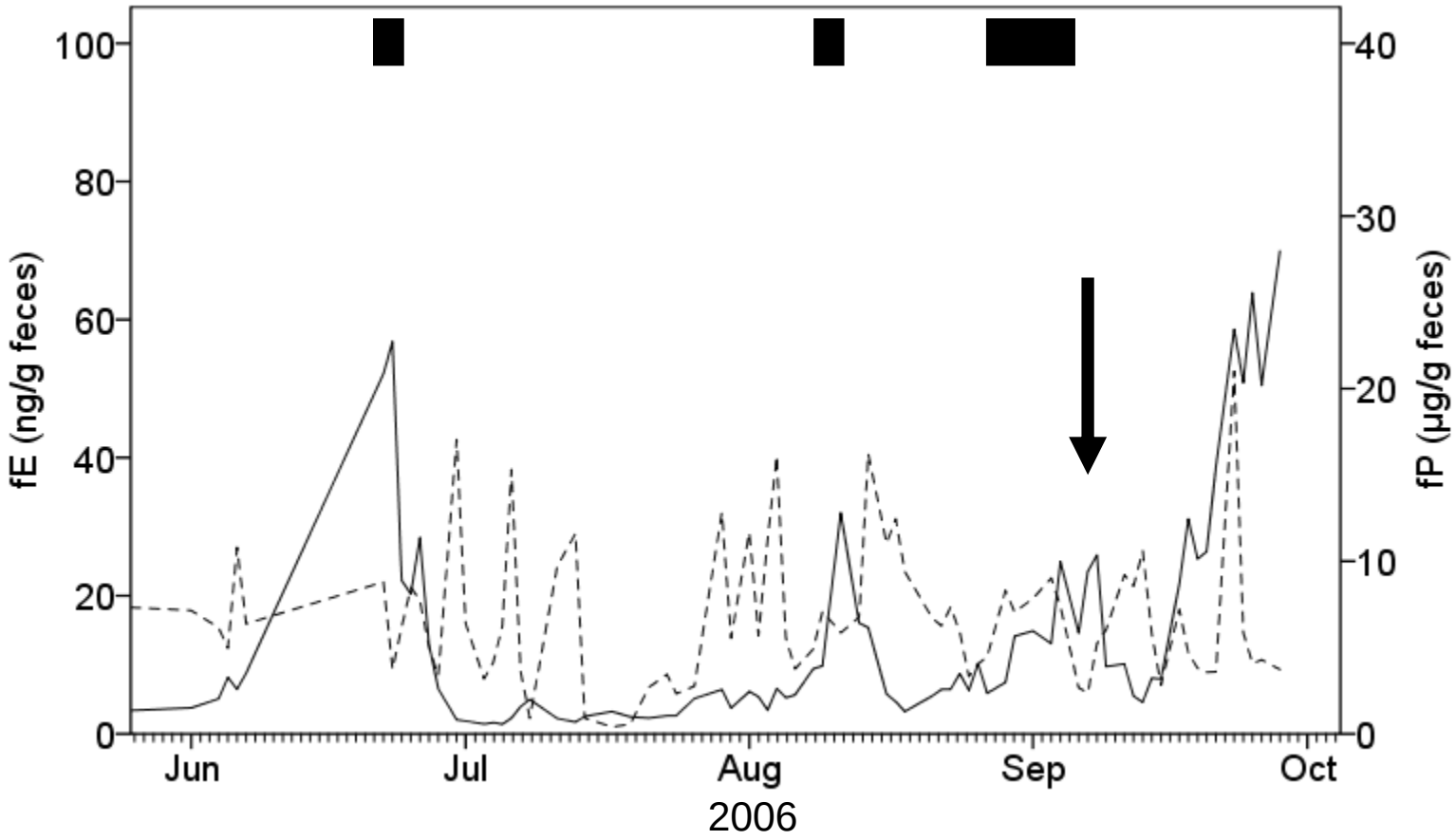

Figure A2.7a Hormone profile for female B9 during entire study period (solid line = fE (ng/g feces); dotted line = fP (µg/g feces); solid arrow = conception date).

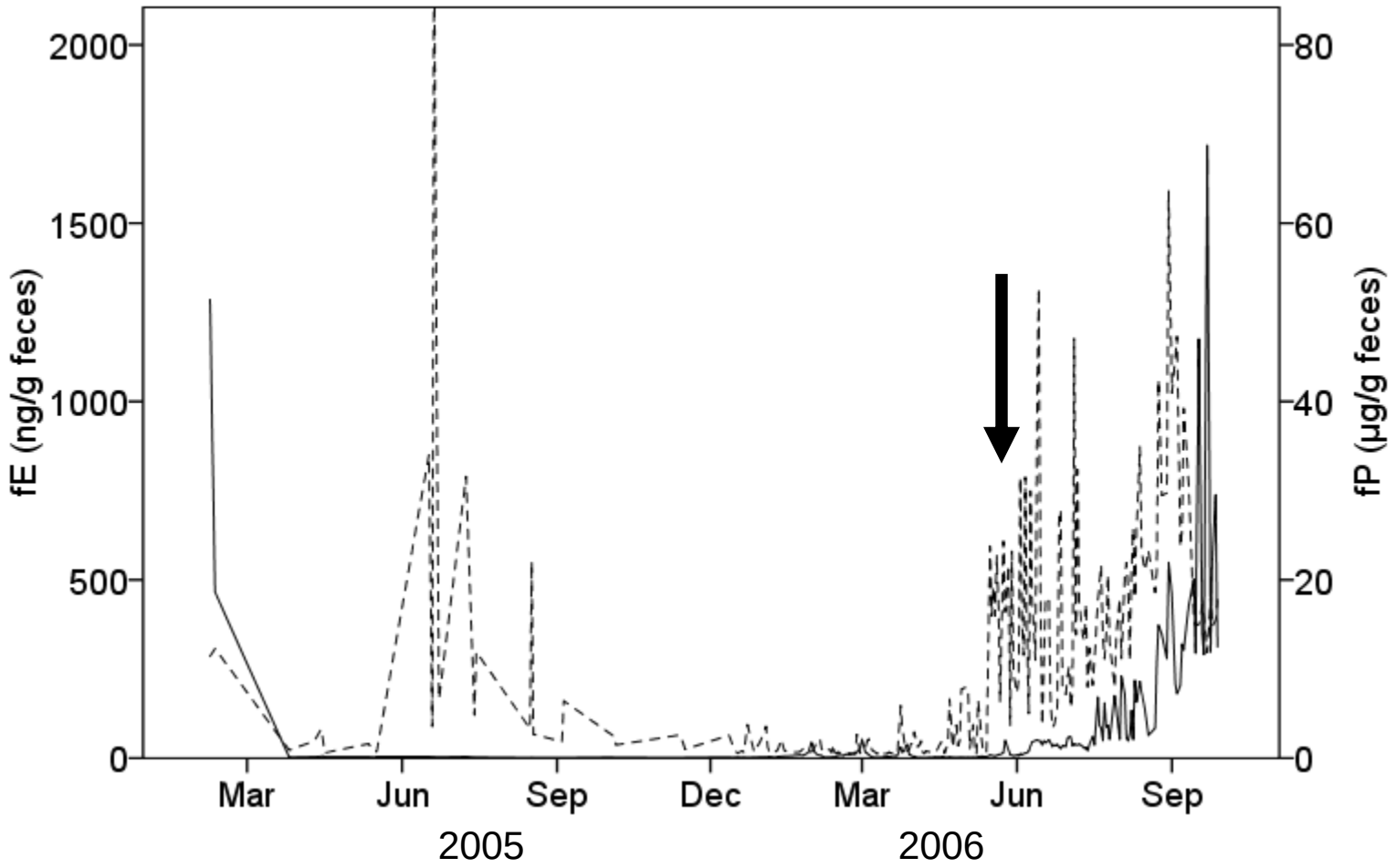

Figure A2.7b Hormone and behavior profile for female B9 prior to conception in 2006 (solid line = fE (ng/g feces); dotted line = fP (μg/g feces); solid arrow = conception date; black horizontal bars = receptive periods).

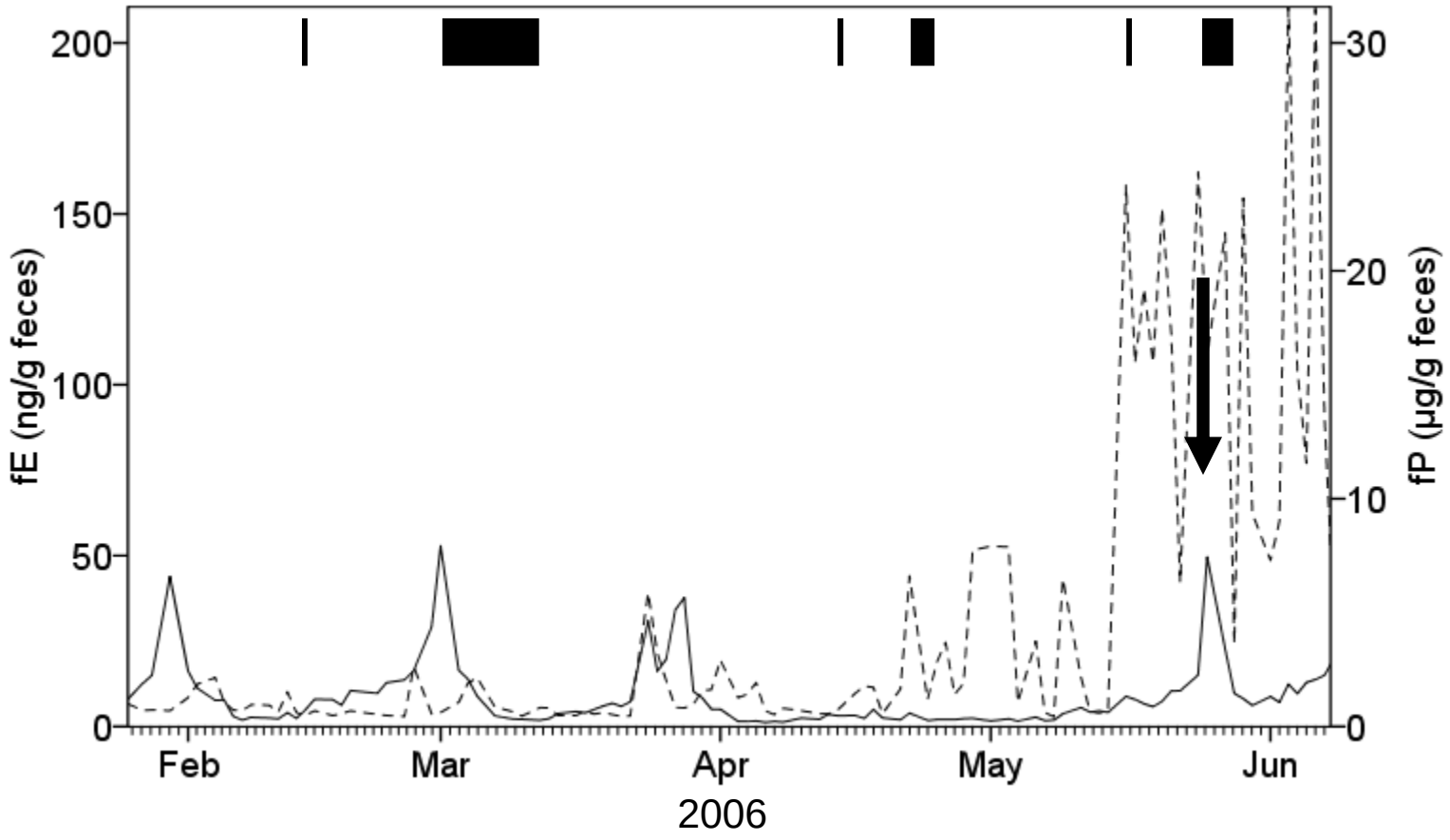

Figure A2.7c Hormone and behavior profile for B9 post-conception in 2006 (solid line = fE (ng/g feces); dotted line = fP (µg/g feces); solid arrow = conception date; black horizontal bars = receptive periods).

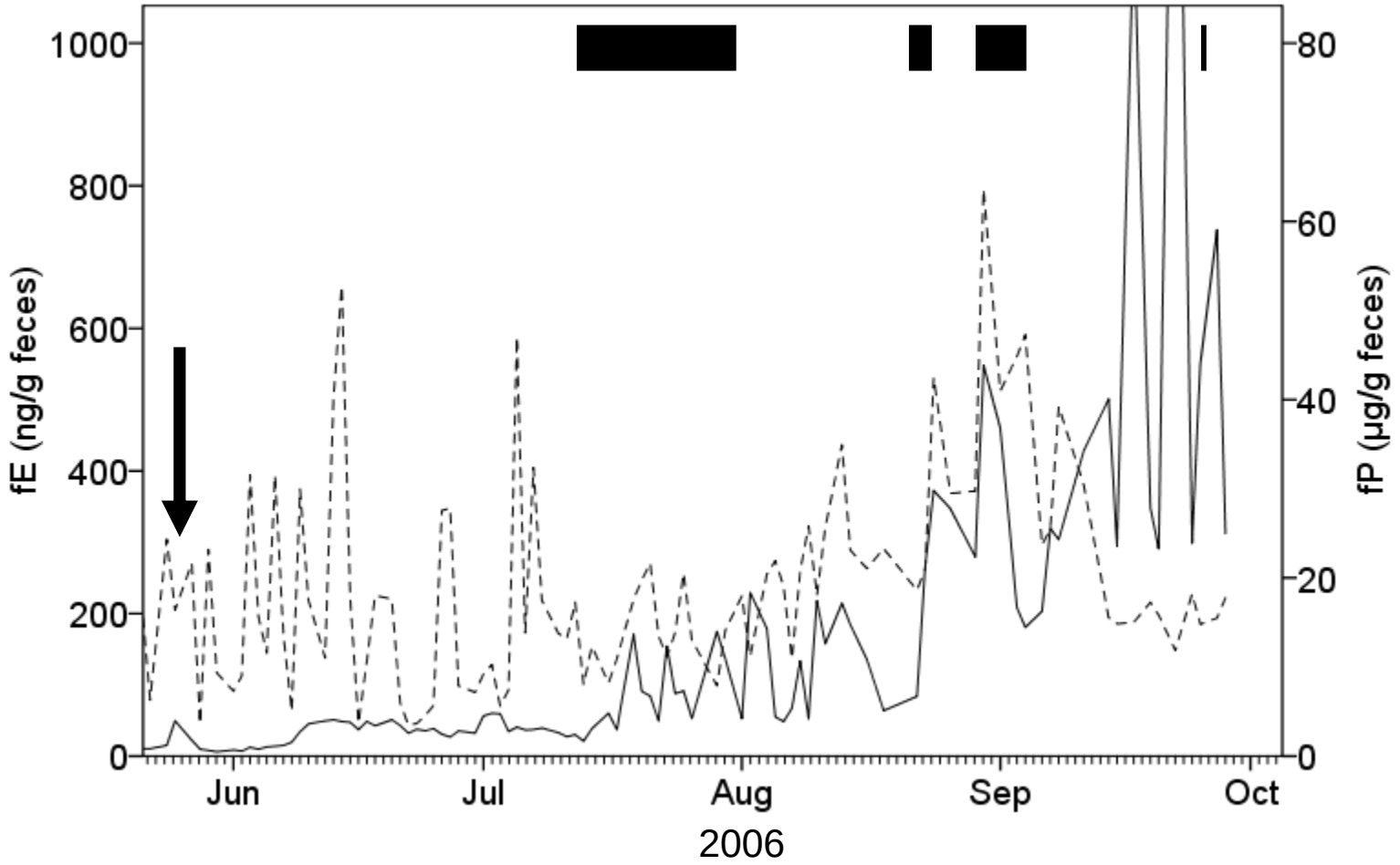

Figure A2.8 Hormone profile for female B10 during entire study period (solid line = fE (ng/g feces); dotted line = fP (µg/g feces); dotted arrow = birth date; solid arrow = conception date).

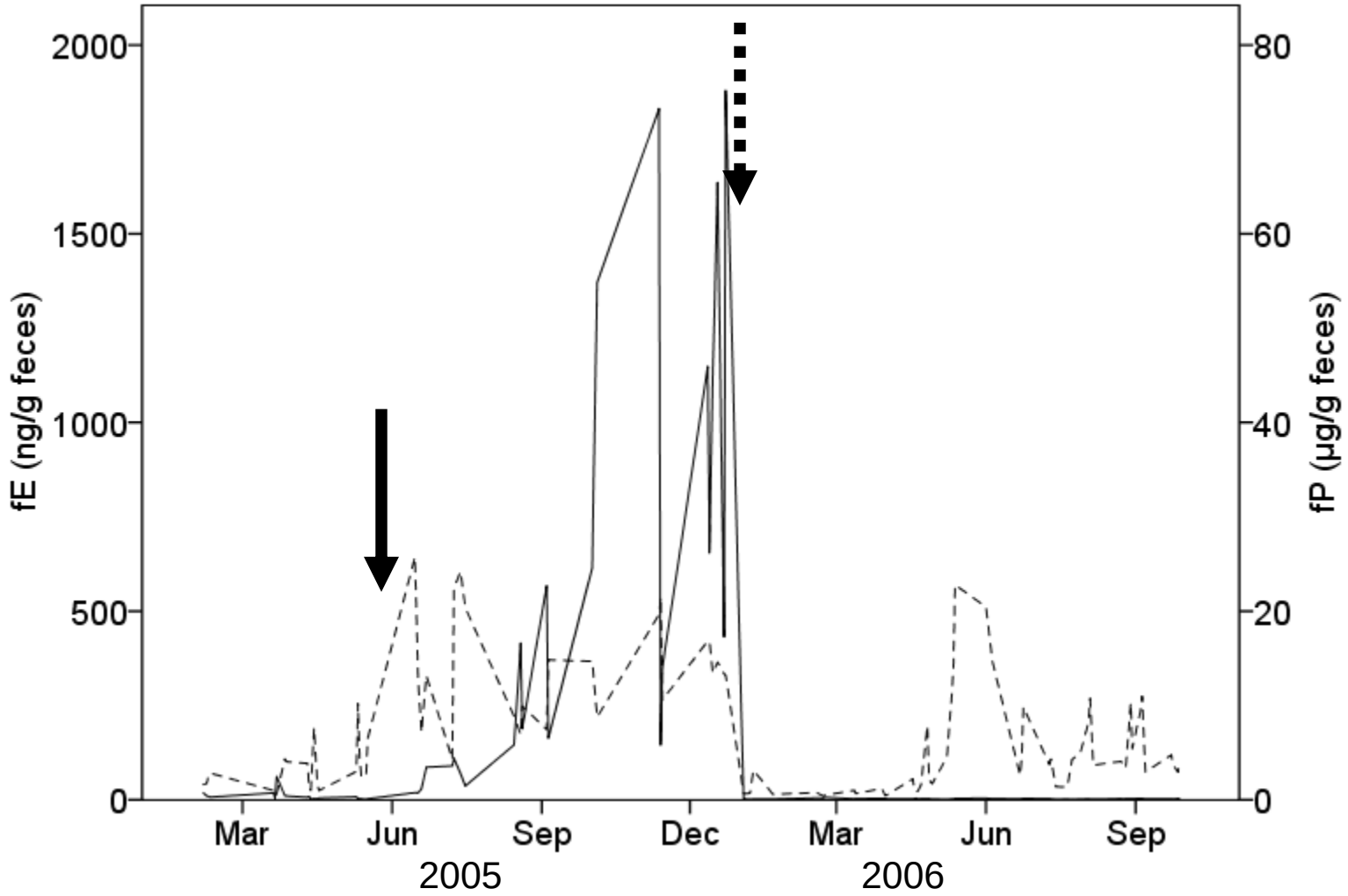

Figure A2.9 Hormone profile for female B11 during entire study period (solid line = fE (ng/g feces); dotted line = fP (µg/g feces); dotted arrow = birth date; solid arrow = conception date).

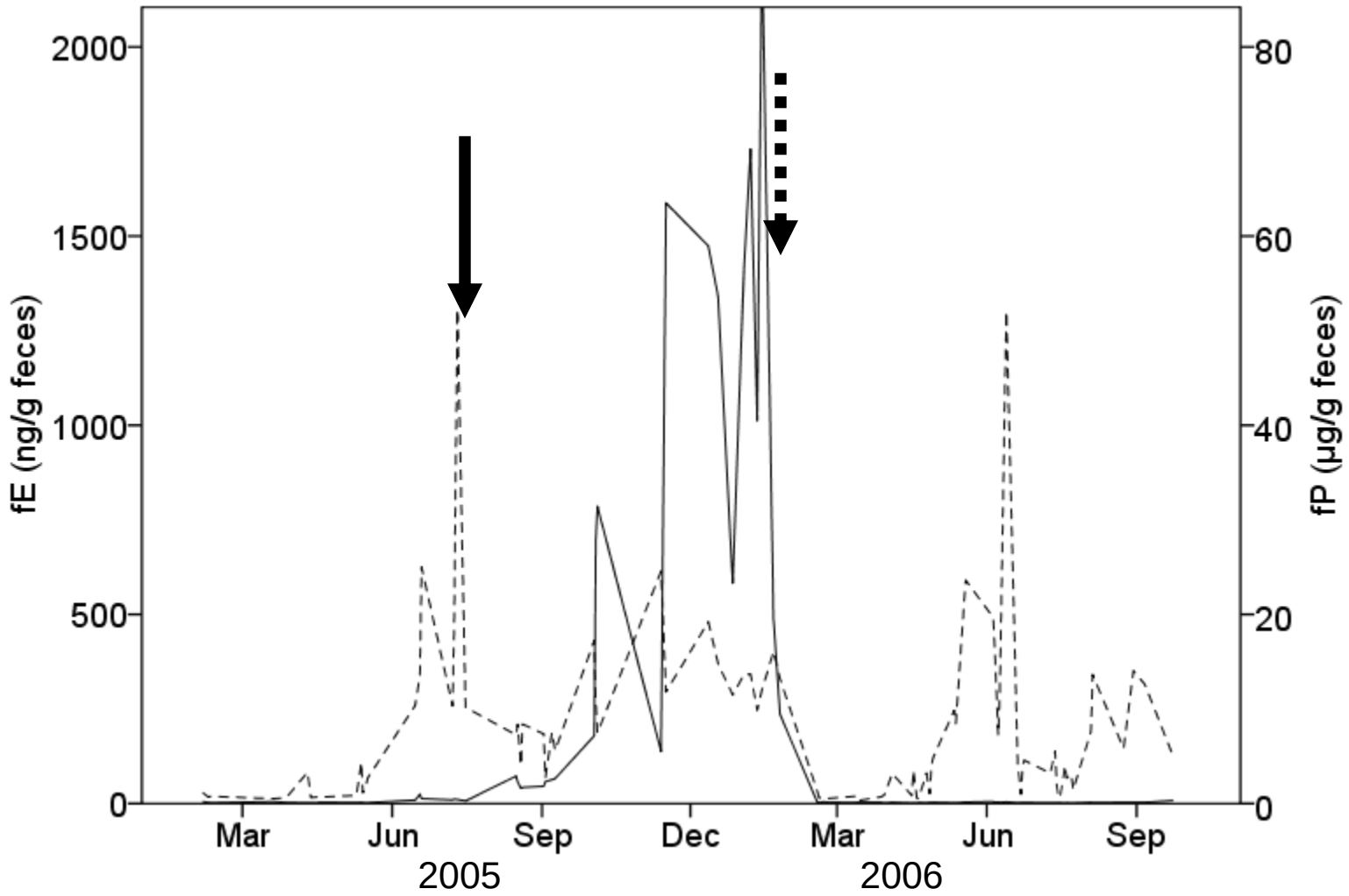

Figure A2.10a Hormone profile for female B12 during entire study period (solid line = fE (ng/g feces); dotted line = fP (µg/g feces); solid arrow = conception date).

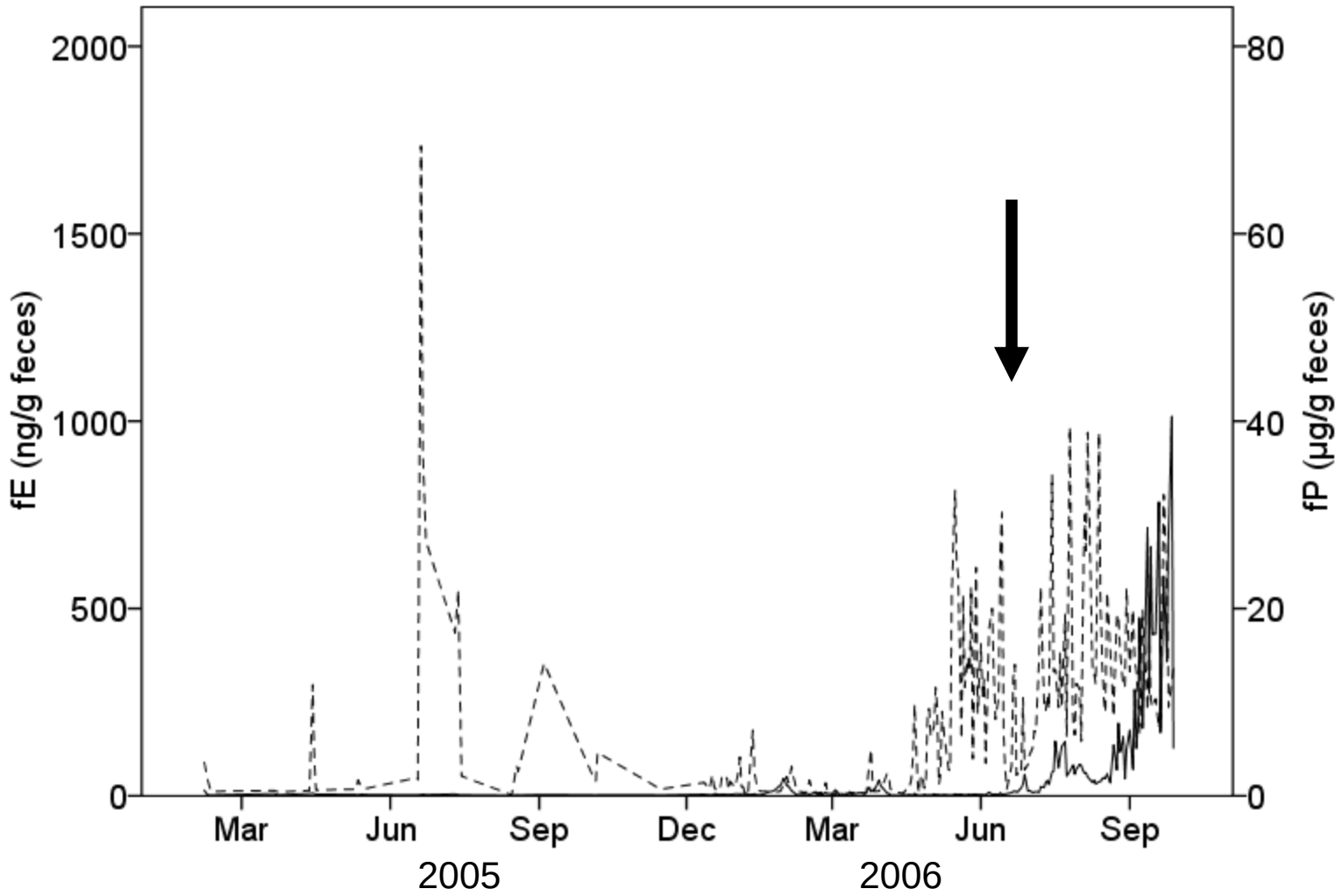

Figure A2.10b Hormone and behavior profile for female B12 prior to conception in 2006 (solid line = fE (ng/g feces); dotted line = fP (µg/g feces); solid arrow = conception date; black horizontal bars = receptive periods).

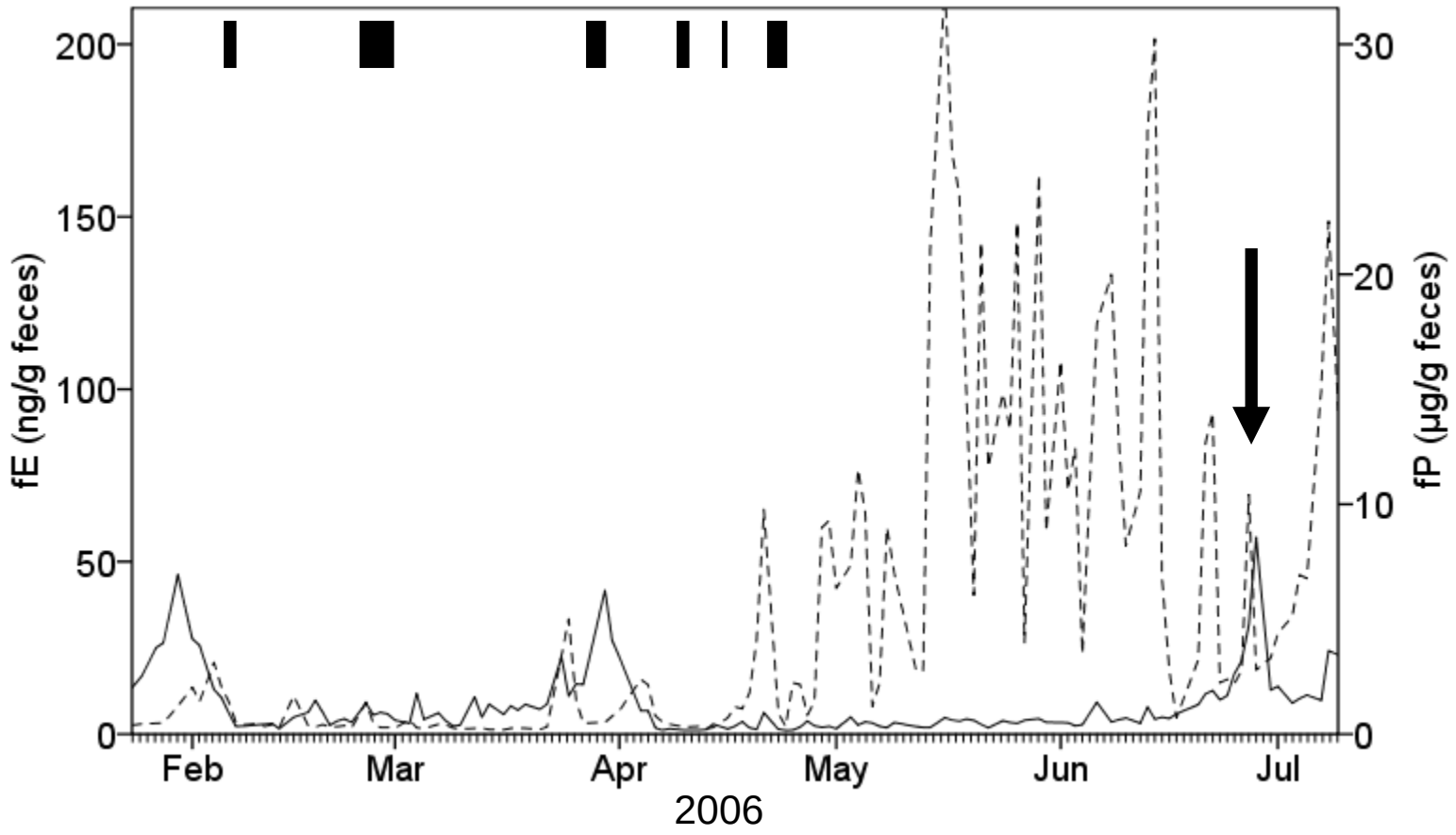

Figure A2.10c Hormone and behavior profile for B12 post-conception in 2006 (solid line = fE (ng/g feces); dotted line = fP (µg/g feces); solid arrow = conception date; black horizontal bars = receptive periods).