Characterization of Follicular Stasis in a Colony of Female Veiled Chameleons (*Chamaeleo calyptratus*)

by

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A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Master of Science
in
Biomedical Sciences

Guelph, Ontario, Canada

ABSTRACT

CHARACTERIZATION OF FOLLICULAR STASIS IN A COLONY OF FEMALE VEILED CHAMELEONS (*CHAMAELEO CALYPTRATUS*)

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This study investigates the etiology, diagnosis, and treatment of follicular stasis in female veiled chameleons (*Chamaeleo calyptratus*). Reproductive status was assessed by enzyme immunoassay of fecal metabolites of estrogen, progesterone, testosterone, and corticosterone; ultrasonography; and male introduction trials. Ultrasonography and hormone pattern analysis confirmed follicular stasis, while female response to male presence was inconclusive. Hormone patterns of corticosterone metabolites indicated a cyclical pattern consistent with reproductive events, but there was insufficient data to compare peak levels between ovulatory and non-ovulatory cycles. Ovulation induction was unsuccessful using either chicken GnRH-II, or a combination of progesterone and prostaglandin F2α. Feed restriction induced weight loss, but this was not directly related to changes in follicle size. Prevention of follicular development (i.e. contraception) was attempted using Depo-Provera and Lupron Depot, but neither treatment was effective. The outcomes of this study supplement the information on follicular stasis in reptiles, but further research is still needed.

ACKNOWLEDGEMENTS

This thesis would not have been possible without the help and contributions of many hard-working people.

Special thanks go out to my project advisor, Gaby Mastromonaco, for providing guidance, knowledgeable advice, patience, always ensuring I had help in the lab and with the chameleons when I needed it, and for providing moral support throughout my project. I am grateful to Drs. W. Allan King, Neil MacLusky, and Gregoy Bedecarrats for being part of my research advisory committee and providing their insight and advice on this project.

I would also like to give special thanks to the Reproductive Physiology staff at Toronto Zoo, in particular, Christine Gilman and Stacey Hayden, for their guidance and assistance with lab work, keeping me motivated, and tolerating my odd sense of humor.

I am very grateful to Christopher Dutton for providing many hours of his time to perform ultrasounds, providing his medical expertise, and friendship.

I would also like to acknowledge the following people for their invaluable contributions:

-Graham Crawshaw; staff veterinarian, Andrew Lentini; animal keeper and Bob Johnson; curator of reptiles and amphibians, for advice on chameleon husbandry and experimental design

-Izzy Hirji, Felicia Listro, and Alyssa Macdonald, co-op and summer students, for taking care of the girls in my absence

-Melissa Felice, fellow graduate student at Toronto Zoo, for your continuing friendship, moral support and helping me out with the girls in times of need

-Charles Guthrie, animal keeper, for his friendship and willingness to help me out with any husbandry-related issues

-Margaret Kolakowski, Nigel Parr, Richard Johnstone, and other members of the Health Unit animal keeping staff for their help taking care of the girls in the mornings and on weekends

-Crystal Hyatt and Danielle Jerome, contract staff and coop student, for your help with the girls and help in the lab

-Michelle Lovering, Dawn Mihailovic,Tasha Beleskey-Long, Pauline Delnatte, and Iga Stasiak, veterinary technicians and veterinary residents for your help providing treatment to the girls

This work was generously funded by the Toronto Zoo, and NSERC.

Robyn H. Pimm
Scarborough, 2013

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DECLARATION OF WORK PERFORMED

I declare that with the exception of the items indicated below, all work reported in the body of this thesis was performed by me.

Some of the enzyme immunoassays (EIA), and assistance with diluting of samples were performed by Christine Gilman, Stacey Hayden, Crystal Hyatt and Danielle Jerome; research assistants in the Reproductive Physiology unit at the Toronto Zoo, under the supervision of Gabriela Mastromonaco. Fecal collections and processing were performed primarily by me, but Crystal Hyatt, and Felicia Listro (co-op student) provided some assistance. All of the ultrasound examinations, administration of drugs, and veterinary treatment were performed primarily by Dr. Chris Dutton; staff veterinarian at the Toronto Zoo, and Pauline Delnatte; veterinary resident, administered some of the treatments. Methods for animal husbandry, and fecal processing were based on those used by Kummrow et al. (2010c,d). All statistical analyses were performed in consultation with William Sears, and Olaf Berke, at the Ontario Veterinary College, University of Guelph, ON Canada.

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INTRODUCTION

Reptiles have inhabited the earth for over 300 million years, and can be found in a variety of habitats in every part of the world, except Antarctica (Bohm et al., 2013; IUCN, 2012; Uetz, 2013). They play a vital role in the maintenance of ecosystems, as both predators and prey, and in seed dispersal and pollination of plants (IUCN, 2012). The class reptilia includes the subgroups amphisbaenia (amphisbaenians), serpentes (snakes), testudines (turtles), crocodylia (crocodiles), rhynchocephalia (tuataras), and sauria (lizards) (Uetz, 2013). It consists of 9,766 species, of which 807 are recognized as threatened (vulnerable, endangered, or critically endangered) on the 2012 IUCN Red List (IUCN, 2012). In addition, a recent global survey of 1500 reptile species indicated that one in five are threatened with extinction; of which 12% were classified as critically endangered, 41% endangered, and 47% vulnerable (Bohm, et. al., 2013). Habitat loss and degradation, climate change, over-exploitation, introduction of alien species, and disease are the major threats to wild animal populations (Bohm et al., 2013; Gibbons, 2000; IUCN, 2012). Although these factors may be difficult to control, a means of preserving wild animal populations at risk of extinction is to involve them in captive breeding and re-introduction programs, both in situ and ex-situ (Snyder et al., 1996).

The American Association of Zoos and Aquariums (AZA) run Species Survival Plan (SSP) programs, which are geared towards the cooperative management of specific, and typically threatened or endangered species populations within AZA-accredited Zoos and Aquariums, certified related facilities, and approved non-member participants (AZA, 2013). These programs ensure the sustainability of healthy, genetically diverse, and demographically varied populations within these institutions that could one day be re-introduced to the wild (if applicable). Breeding and transfer plans are developed based on comprehensive population studbooks, with consideration of the species' social and biological needs, as well as transfers feasibility. The need to transfer breeding males and females between different institutions is one of the drawbacks of these programs, as many institutions have a minimum quarantine requirement before the animals can be transferred out, or introduced to a new population. This can postpone breeding, which can have deleterious effects to the reproductive health of females.

In some species of reptiles, females will continue to cycle and produce (non-viable) eggs in the absence of a male. Egg-production is an energetically costly process and puts a strain on females' bodies, and thus is undesirable if offspring are not being produced. In addition, reproductive health disorders are common in captive female reptiles. The most common condition is known as egg-binding; by definition, it is the retention of eggs within the reproductive tract beyond the length of a normal cycle (Cuadrado, 2000; DeNardo et al., 2002; Rivera, 2008; Sykes, 2010). This term is used to describe both *follicular stasis*; the failure to ovulate and subsequently reabsorb yolk from follicles, and *dystocia*; the failure to lay fully formed eggs. Follicular stasis has resulted in the deaths or loss of breeding potential (due to removal of the reproductive tract) in many captive female reptiles (Rivera, 2008; Stacy, 2008; Sykes, 2010). The etiology is not well understood, but the presence or absence of a male at various stages of the reproductive cycle, and environmental factors have been implicated as possible causes.

Therefore, developing a better understanding of the causes, and methods to prevent or treat this condition would be valuable to prevent the loss of breeding females and increase the success of captive breeding efforts.

REVIEW OF LITERATURE

REPTILES IN CAPTIVITY

The number of reptiles in captivity is increasing. They are becoming more popular as household pets, in animal collections in zoos and related attractions for display and educational purposes, and many endangered and threatened species are being captive bred as part of co-operative breeding and re-introduction programs among different institutions (DeNardo et al., 2002; Kramer, 2006; Rivera, 2008; Snyder, et al., 1996).

Although specific animal husbandry guidelines have been developed for animals in captivity (based on wild counterparts), there remain a few challenges which can affect the long-term health and well-being of the animals. It is difficult to replicate seasonal variations in temperature, humidity, photoperiod, and prey/food availability which regulate behavioural and physiological events. This is particularly challenging in an institution, such as a zoo, where there is the necessity of having animals on display and visible to visitors throughout the day year-round. As a result, timing of hibernation and emergence (when applicable), the onset or recrudescence of (different stages of) the reproductive cycle, and courtship, mating, and nesting behaviours may be negatively affected (DeNardo, 2002; Greenberg and Wingfield, 1987; Rivera, 2008; Summers, 1988).

Many female reptiles in captivity cycle year-round (rather than seasonally), and produce larger clutches, with larger sized eggs compared to wildlife counterparts as a result of being kept in unvarying conditions (Cuadrado, 2000; DeNardo et al., 2002; Rivera, 2008). Many factors regulate follicle recruitment and clutch size in reptiles, including female body size, circulating levels of follicle stimulating hormone (FSH), and the degree of ovarian vascularity, (Mendez-de la Cruz et al., 1993). However, environmental conditions can have a drastic impact on the number of follicles that continue to develop or undergo atresia (DeMarco, 1989; Mendez-de la Cruz et al., 1993; Summers et al., 1998). *Anolis carolinensis* held under chronic low-humidity (<30%) conditions had severely depressed ovarian and oviductal growth in comparison to those held under normal conditions (>60% humidity) (Summers et al., 1998). In addition, DeMarco (1989) noted that egg size was negatively related to clutch size in *Scleroporus woodi* during a year of severe drought. Further, body condition and resources available at the time of

vitellogenesis may impact egg size (DeMarco, 1989; Rhen et al., 2006; Shanbag et al., 2000).

Rhen et al. (2006) state that the energy required for egg production can come from stored resources (capital), those acquired during reproduction (income), or a combination of both; and is proportional to the energy available. Females in captivity acquire a constant source of income energy, and are thus able to put more resources into egg production. Conversely, in theory, if a female is in a negative energy state and is unable to acquire additional income energy, she will down-regulate egg production in order to conserve resources. This phenomenon has been observed in laying hens; molting induced by feed restriction results in the hens shedding their feathers, and follicular regression occurring on the ovary (Koch, 2005). As a result, egg-laying is put off until the molt is complete.

Population management of captive populations may require that breeding be postponed due to delays in acquiring males or females from other institutions, compatibility problems, or space/housing restrictions (Snyder et al., 1997). In some egg-laying species, females will continue to cycle and produce non-fertile eggs in the absence of a male (Rivera, 2008), such as the commonly kept green iguana; *Iguana iguana* (DeNardo et al., 2002) and the veiled chameleon (Kummrow et al. 2010c,d) while in others, male presence is necessary for normal reproductive events to occur, as seen in red-sided garter snakes; *Thamnophis sirtalis parietalis* (Mendoca and Crews, 1990) blood pythons; *Python curtus* (DeNardo and Autumn, 2001) and anole lizards; *A. carolinensis* (Crews, 1975).

Parthenogenesis, the production of viable offspring in the absence of fertilization by a male has been reported in some reptile species, including the Komodo dragon; V*aranus komodoensis* (Sunter, 2008; Watts et al., 2006), Argus monitor lizard; V*aranus panoptes* (Lenk et al., 2005) and several species of whiptail; *Cnemidophorus* lizards (Moore et al., 1985). However, this mechanism of reproducing is undesirable since the offspring would be genetically homozygous, and in the case of Komodo dragons, all male (Watts et al., 2006).

Altogether, husbandry conditions can have a dramatic impact on egg-production and reproductive health of animals.

FEMALE REPTILE REPRODUCTION

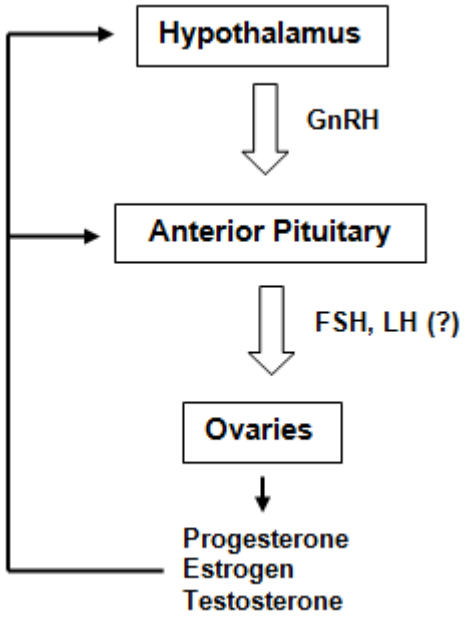
There are two main modes by which female reptiles reproduce; viviparity and oviparity

(Adams et al., 2007; Blackburn, 2000; Kummrow et al., 2010c,d). Viviparity applies to species that give birth to live offspring (Adams et al, 2007; Blackburn, 2000; Dauphin-Villemant et al., 1990; Yaron and Widzer, 1978). Embryos develop within the maternal reproductive tract, where they derive nourishment through a placenta or similar structure. This mode is used by the viviparous or common lizard; *Lacerta vivipara* (Dauphin-Villemant et al., 1990), desert night lizard; *Xantusia vigilis* (Yaron and Widzer, 1978), and several species of skinks; *Scincidae sp.* (Jones and Swain, 2000; Swain and Jones, 1994), among others. In contrast, oviparity refers to species that produce and lay eggs (Adams et al., 2007; Kummrow et al., 2010c,d). Little or no embryo development occurs within the mother, and nutrients are provided from surrounding yolk. Some examples of oviparous reptiles include the common side-blotched lizard; *Uta stansburiana* (Wilson and Wingfield, 1992), green anoles; *A. carolinensis* (McNicol and Crews, 1979; Jones et al., 1988), and several species of chameleon; *Chamaeleo sp.* (Cuadrado, 2002; Kummrow et al., 2010c,d). For the purpose of this review, the focus will be on the oviparous mode of reproduction.

The patterns of ovulation in reptiles have been classified as polyautochronic, monoautochronic, and monoallochronic (Etches and Petitte, 1990). Polyautochronic ovulation refers to the simultaneous ovulation of many follicles from both ovaries, which is seen in most species; monoautochronic denotes the simultaneous ovulation of one follicle from both ovaries, which is common in geckos; and monoallochronic is where only one follicle is ovulated at a time from each ovary, alternating between each single egg clutch as seen in *A. carolinensis* (Jones et al., 1979).

The reproductive cycle of oviparous reptiles consists of five stages; previtellogenesis, vitellogenesis, ovulation, gravidity, and oviposition (Kummrow et al., 2010c; Moore and Crews, 1986; Rhen et al., 2000). However, in the absence of ovulation, follicles undergo atresia and the yolk is reabsorbed. The stages of the reproductive cycle are mediated by reproductive hormones produced by the hypothalamic-pituitary-gonadal (HPG) axis (Figure I) (Licht, 1979). Gonadotropin releasing hormone (GnRH), is produced and released from the hypothalamus, and binds to receptors on the pituitary gland to stimulate the release of FSH from the anterior pituitary (Licht, 1979). FSH travels through the systemic circulation and binds to receptors on the gonads (testes or ovaries) and elicits physiological effects, which stimulates the production and release of the steroid hormones: estrogen, progesterone and testosterone.

Figure I Hypothalamic-pituitary-gonadal (ovarian) axis



Hormones of the hypothalamic-pituitary-gondal (HPG) (ovarian) axis. GnRH: gonadotropin releasing hormone; FSH: follicle stimulating hormone; LH: luteinizing hormone.

During the first stage of the cycle, previtellogenesis, small primordial follicles can be found on the ovaries (Jones et al., 1976; Kummrow et al., 2010c,d). Under the influence of increasing amounts of FSH secreted by the anterior pituitary, follicles enter vitellogenesis, the period of rapid follicular growth, when yolk gets deposited into the follicles (Carnevali, 1991; Ho et al., 1992; Yaron and Widzer, 1978). Growing follicles secrete large amounts of estrogen, and this stimulates the production of vitellogenin from the liver (Carnevali et al., 1991; Ho et. al., 1982; Licht, 1979; Moore and Crews, 1986; Sykes, 2010; Yaron et al 1978). The yolk is mobilized from the liver, and transported via the blood to the ovary where it is incorporated by the developing oocytes. Once the follicles have reached maturity, they are expelled from the ovary, the process known as ovulation. In mammals, the trigger for ovulation is a surge in circulating levels of luteinizing hormone (LH); however, a gonadotroph with LH-like actions has not yet been identified in reptiles (Licht, 1979; 1984). The specific trigger for ovulation in reptiles remains unclear, although it has been proposed that FSH may also play a role in the process (Licht, 1984). It has also been proposed that progesterone may trigger ovulation in bearded dragons, *Pogona barbatta* (Amey and Whittier, 2000). Following ovulation is the gravid period, during which fertilization occurs (if applicable), corporal lutea form and begin to secrete

progesterone, and eggs can be found along the length of the oviducts (Feldman, 2007; Kummrow et al., 2010c; Shanbag et al., 2001; Van Wyk, 1994). Eggs remain in the oviducts for a species-specific amount of time, until they acquire a calcified shell, after which they are oviposited. Prior to oviposition, progesterone levels fall, allowing uterine contractions to occur.

In some species of reptiles, females can retain sperm in the reproductive tract for months and still produce viable offspring (Ortega-Leon et al., 2009; Shanbag et al., 2001; Wolf, 2009); therefore, mating does not have to occur immediately prior to ovulation. For example, Ortega-Leon et al. (2009) reported that sperm stored in the reproductive tract of female cleft lizards; *Scleroporus mucronatus* were able to fertilize eggs after 4 months, and one study reported the retention of sperm for 430 days in a female eublepahrid gecko; *Aeluroscalabotes felines* (Wolf, 2009).

EGG-BINDING

A condition known as egg-binding or egg retention is the most commonly observed reproductive disorder affecting captive female reptiles (Cuadrado, 2002; Kramer, 2006; Rivera, 2008; Sykes, 2010). By definition, it is the retention of eggs within the reproductive tract beyond the length of a normal cycle. It can occur at the level of the ovary, when follicles fail to ovulate and subsequently do not recede normally; known as *follicular stasis*, or in the oviduct, when fully formed eggs are not laid; known as *dystocia*. Retained eggs can cause inflammation, fluid build-up, compaction of the digestive tract, and carry a high risk of rupture which can lead to a condition known as egg yolk coelomitis, and, if left untreated, can result in death of the animal (Rivera, 2008). Yolk coelomitis has been identified as a major cause of death in captive Komodo dragons (*Varanus komodoensis*) (Spellman, 2002 as cited by Sykes, 2010), and Fiji Island banded iguanas (*Brachylophus fasciatus*) (Stacy et al., 2008).

The etiology of this condition is not well understood. Some of the speculated causes are stress, malnutrition, lack of a proper nesting site, and the presence or absence of a male, which is both species- and stage of cycle-specific (Backues and Ramsay, 1994; Kramer, 2006; Sykes, 2010). Male displays and courtship have been identified as an important factor for normal ovarian development and ovulation in iguanas (Dugan, 1982 as cited by Backues and Ramsay, 1994), and the rate of environmentally induced ovarian recrudescence and egg shell formation in female *A. carolinensis* (Crews, 1975). It is hypothesized that male presence affects

gonadotropin production and release, and this facilitates normal reproductive processes (Crews, 1975).

Preventative measures have been limited to surgical removal of parts or all of the reproductive tract (Kramer, 2006; Rivera, 2008; Sykes, 2010). This is a good option when the animal is not required for breeding, but far less desirable for animals meant to be involved in breeding programs. Contraception has been attempted in reptiles, but with varying results and limited success, and there is no current treatment/method available (discussed further in a later section).

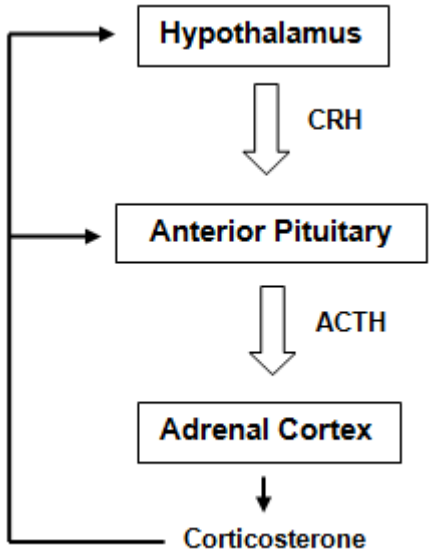
Although dystocia can be readily treated (and often resolved) with the administration of arginine vasotocin (AVT) or oxytocin to induce oviposition, treatment of follicular stasis has been limited to individual follicle aspiration, a perilous procedure which carries a high risk of follicle rupture, and fails to prevent future occurrences (Rivera, 2008). To the author's knowledge, no attempts at ovulation induction for the treatment of follicular stasis have been reported.

The early recognition of the condition and distinguishing between pre- or post-ovulatory egg retention is critical for developing a treatment plan, and the prevention of subsequent pathologies (Kramer, 2006).

THE HPA AXIS IN REPTILES

In response to a stressor/stimulus, the hypothalamic-pituitary-adrenal (HPA) axis gets activated (Johnson et al., 1990; Moore and Jessop, 2003; Palme et al., 2005). Corticotropin releasing hormone (CRH) is produced and secreted by the hypothalamus, and triggers the release of adrenocorticotropin hormone (ACTH) from the anterior pituitary (Figure II). ACTH then travels through the systemic circulation, binds to receptors on the adrenal glands, and stimulates the production and release of glucocorticoids from the adrenal cortex. In reptiles, the main glucocorticoid metabolite produced is corticosterone (CORT), in contrast to cortisol in mammals (Moore and Jessop, 2003; Wilson and Wingfield, 1992).

Figure II Hypothalamic-pituitary-adrenal axis



Hormones of the hypothalamic-pituitary-adrenal (HPA) axis. CRH: corticotropin releasing hormone; ACTH: adrenocorticotropic hormone.

STRESS AND REPRODUCTION

Animals respond to stress by modifying their behaviour, mediated by physiological changes, in order to increase the chances of survival. This phenomenon is known as the "general adaptation syndrome" (Moore, and Jessop, 2003; Johnson et al., 1990). This may include suppression of reproductive behaviour and down-regulation of the HPG axis by the production of hormones through the HPA axis (Johnson et al., 1992; Moore and Palme, 2002; Palme et al., 2005).

Inhibition can occur at various locations of the HPG axis. At the level of the hypothalamus, CRH directly inhibits the release of GnRH (Johnson et al., 1992; Palme et al., 2005). Production and release of FSH are reduced as a result of decreased amounts of GnRH, and are also directly inhibited by ACTH and corticosterone. Gonadal activities (such as folliculo/vitellogenesis and ovulation) are subdued, and sex steroid hormone production and release is reduced in response to lower circulating levels of FSH. Additionally corticosterone and progesterone produced by the adrenal glands inhibit estrogen production from the ovaries,

and prevent oviductal contractions in gravid animals (Shanbhag et al., 2001; Woodley and Moore, 2002). This can result in a reduction in estrogen-driven behaviours and inhibition of physiological events, such as vitellogenesis and oviposition. For example, Shanbhag et al. (2001) found that oviductal contractions were inhibited by elevated levels of progesterone in stressful conditions in *Calotes versicolor*. In contrast, Woodley and Moore (2002) did not find elevated levels of progesterone in response to an acute stressor in gravid tree lizards; *Urosaurus ornatus*, which suggests that stress is not responsible for inhibiting oviductal contractions in that species.

Summers (1988) and Summers and Norman (1988) examined the effects of chronic low humidity on adrenal gland activity and ovarian recrudescence in *A. carolinensis*. Animals kept in low-humidity conditions exhibited peaks of corticosterone throughout the day (compared to control and high-humidity groups which only had one peak), were more often brown in colour rather than green (an indicator of stress in this species), and exhibited retarded ovarian growth, among other body condition traits. In addition, altering environmental conditions towards those which stimulate ovarian and oviductal growth; i.e. high temperature and longer photophase, can shift the diurnal rhythm of corticosterone release. Nijagal and Yajurvedi (1999) found that the administration of corticosterone to keeled Indian mayuba; *Mayuba carinata* inhibited FSH-induced (vitellogenic) follicular growth, and decreased circulating estradiol concentrations compared to lizards treated with only FSH. From these data, they speculated that stress-induced corticosterone secretion would act to inhibit vitellogenesis as an energy-saving mechanism.

Although the relationship between stress and reproduction is often perceived to be negative, increased levels of glucocorticoids may actually mediate or be a consequence of normal reproduction (behaviourally and physiologically) (Moore and Jessop, 2003). Woodley and Moore (2002) found that corticosterone was positively correlated with ovarian weight in vitellogenic female tree lizards (*U. ornatus*), and proposed that it may have a facilitative role in the reproductive cycle of this species. Wilson and Wingfield (1992) found similar results in side-blotched lizards (*U. stansburiana*), with corticosterone levels increasing with vitellogenesis, and remaining elevated during the gravid period. It was proposed that increased corticosterone production during vitellogenesis was related to increased levels of plasma estradiol which caused enlargement of the adrenal glands in the desert iguana *Dipsosaurus dorsalis* (Callard and Callard, 1978 as cited by Woodley and Moore, 2002). Gobbetti et al. (1995) found adrenal

production, and plasma levels of corticosterone were highest during ovulation in the lizard *Podarcis sicula sicula*, and suggested that it may play a role in inducing ovulation in this species. In contrast, Grassman and Crews (1990) found that corticosterone levels were highest during pre-vitellogenesis, declined during vitellogenesis, and remained at pre-ovulatory levels during gravidity in the parthenogenetic oviparous whiptail lizard (*C. uniparens)*.

However, several studies have demonstrated a lack of relationship between levels of corticosterone and different stages of the reproductive cycle. For instance, in studies of female common geckos; *Hoplodactylus maculates* (Girling and Cree, 1995), and female bearded dragons; *P. barbata* (Amey and Whittier, 2000), no significant differences in plasma corticosterone levels were found between different stages of the reproductive cycle.

The relationship between the adrenal and reproductive axes is complex and varies from species to species. In some it appears to be facilitative or necessary for reproductive events to occur, whereas in others it does not appear to have any relationship to the reproductive cycle. Therefore it is not possible to universally define this relationship in reptiles.

MANIPULATION OF REPRODUCTIVE CYCLE IN REPTILES AND RELATED SPECIES

A few studies have investigated the possibility of hormonal manipulation of ovarian processes in reptiles; including ovulation induction and inhibition of follicular growth.

Ovulation Induction

Ovulation induction of ripe follicles has been successfully accomplished using mammalian and reptilian (turtle) variations of FSH and LH, alone or in combination in a variety of lizard species, including: *A. carolinensis* (Jones et al., 1988; Jones et al., 1990; Licht 1970; Licht and Crews, 1975; Licht and Tsui, 1975), garden lizards; *C. versicolor* (Shanbag and Prasad, 1993), common side-blotched lizards; *U. stansburiana* (Licht, 1970), Texas horned lizards; *Phrynosoma cornutum* (Burns and Richards, 1974), and western fence lizards; *Sceloporus occidentalis (*Licht, 1970). All studies on lizards have shown that the potency of LH is minimal compared to FSH in stimulating ovarian and oviduct growth, and there is only one report of ovulation induction from the administration of (turtle) LH in *A. carolinensis* (Licht and

Crews, 1975). In contrast, Callard et al. (1976) and Chan and Callard (1974) found that mammalian LH had substantially more potent stimulatory effects compared to mammalian FSH on *in vitro* steroidogenesis in ovarian tissue from painted turtles (*Chrysemys pitta*) and cooters (*Pseudemys*), respectively. Similarly, Licht (1972) found that ovarian activity in *C. pitta* was not stimulated by ovine FSH *in vivo.* In contrast, Lance and Callard (1978) measured plasma levels of sex steroids in response to ovine FSH and LH in *C. pitta* and the southern water snake; *Natrix fasciata,* and found that FSH had more potent effects than LH in the turtles, but similar responses were seen to both gonadotropins in the snake (although plasma levels of hormones were only measured up to 48 hrs and stage of follicular development varied considerably).

All of the aforementioned studies examined the effects of gonadotropins on healthy ovaries/tissues and follicles of reptiles; thus far, there have been no attempts at inducing ovulation in egg-bound animals.

Although both FSH and LH-like hormones have been isolated from the turtle pituitary, no LH-like hormone has been isolated from lizards or snakes (Licht, 1984). However, Aizawa and Ishii (2003) determined the full-length cDNA encoding the LH β subunit precursor molecule of the Japanese grass lizard (*Takydromus tachydromoides*) and deduced the final amino acid sequence of the mature peptide (referring to the amino acid sequence of the bullfrog LH β subunit molecule). The amino acid sequence of the mature peptide was compared with those of other vertebrates, and found to be more closely related to amphibian LH β subunit molecules than to avian or mammalian LH β subunit molecules (Aizawa and Ishii, 2003). Further, they compared the two receptor interacting domains (i.e. the large and determinant loops in the gonadotropin molecule) of the lizard LH β with avian LH β and mammalian FSH β and found a high degree of similarity between the characteristic residues. Extrapolating from results of a study by Miya et al. (1994) (as cited by Aizawa and Ishii, 2003) they predict that mammalian FSH receptors recognize reptilian LH, and reciprocally, that reptilian LH receptors recognize mammalian FSH. To the author's knowledge, no studies have examined the expression of the LH β encoding gene throughout different stages of the reproductive cycle, nor has the genetic sequence of the LH receptor in reptiles been characterized.

Studies on amphibians, including the Indian skipper-frog (*Rana cyanophlyctis*) and northern leopard frog (*Rana pipiens*) have shown that progesterone is capable of inducing ovulation *in vitro* (Edgren and Carter, 1963; Ramaswami and Lakshman, 1958; Wright, 1961 &

1971). The effect of exogenous progesterone on ovulation has not been studied in lizards.

Hormone Contraceptives

Hormone contraceptives act by shifting endogenous hormone production and release by altering or disrupting the HPG axis feedback mechanisms (Herbert and Trigg, 2005; Rivera et al., 1999). They act directly on any hormone or organ within the HPG axis. Ultimately, the release of gonadotropins from the pituitary and sex steroids from the reproductive organs is altered in such a way that normal reproductive events such as folliculogenesis, ovulation, conception, and implantation/gestation are prevented.

Physical characteristics of the reproductive tract may also be altered; for instance, increasing the viscosity of the vaginal mucus, decreasing the build-up of the lining of the uterus and reducing oviduct weight (Rivera et al., 1999).

Modes of action

There are three modes of hormonal contraception: agonists, antagonists, or immunization (Herbert and Trigg, 2005). Agonists are similar in structure to native, endogenous hormones, but are more stable (possess a longer half-life), and exert a stronger, more profound physiological response. If high levels are maintained for an extended period of time, down-regulation of the hormone receptors will occur. Antagonists are similar in structure to native hormones but do not exert any physiological function. Instead, they compete for hormone receptors, thereby inhibiting binding of the native hormone; eliminating any downstream physiologic effects. Immunization involves administering a vaccine that induces an adaptive immune response, which results in the animal making antibodies against its own native hormone. The binding of the antibody changes the shape of the hormone and inhibits binding to receptors. In addition to being very costly, this method often requires booster shots, and produces variable/inconsistent results within and between individuals of a given species.

Hormones analogues

The most commonly used hormone analogues for contraception are progesterone,

GnRH and estrogen.

Progesterone

Limited literature exists on the use of progesterone analogues to induce physiological changes in reptiles. They vary in the location and/or route of administration, concentration, dosage, and effects observed. Callard et al (1972a) found that injections of progesterone (dose 2 mg/ 0.1 ml sesame oil) daily for 14 or 28 days inhibited follicular growth in *Sceloporus cyanogenys*, induced some follicular atresia, retarded ovarian growth and prevented ovulation in eight out of nine animals when administered during vitellogenesis. Interestingly, they found that it had no effect early in the breeding season, during pre-vitellogenesis. In that same study, it was observed that progesterone implanted subcutaneously or intrahypothalamically did not alter ovulation frequency. In a study by Callard and Doolittle (1973), blue spiny inguanid lizards; *S. cyanogenys* implanted with a subcutaneous progesterone implant for 45 days showed no effect on ovarian growth, and seven out of 8 females ovulated normally. However, when the animals received an implant in the hypothalamus they observed atrophy of follicles and the oviduct.

Yaron and Widzer (1978) examined the anti-gonadal effects of progesterone by injecting female *X. vigilis* with progesterone daily for 21 days. They observed a significant reduction of ovarian weight and of follicular diameter when compared to control animals. They also describe another experiment (unpublished) in which they attached silicone implants containing progesterone unilaterally to the ovaries of *Lacerta sicula* and treated the animals with pregnant mare serum (PMS) to induce ovarian activity. The results showed retarded growth on the ovaries containing the progesterone implant compared to the contralateral ovaries implanted with silicone only.

GnRH

Treatment with low doses of GnRH analogues has a stimulatory effect, inducing a large increase the production and release of FSH and LH from the pituitary gland, and was initially used to treat infertility (Herbert and Trigg, 2005; Schneider et al., 2006). However, a high dose or chronic administration results in a refractory effect; whereby GnRH receptors are down-regulated and there is desensitization of pituitary gonadotropes.

No studies to date have been published that examined the effectiveness of GnRH as a method of contraception in reptiles. However, the use of deslorelin as a means of contraception in female veiled chameleons is currently being studied (personal communication, Dr. Zdenek Knotek, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic), but no results are available at this time.

Studies indicate that the GnRH decapeptides found in reptiles are identical in structure to the two forms found in birds (chicken GnRH; cGNRH I and II), as opposed to the GnRH forms found in mammals (mammalian GnRH) or fish (salmon GnRH) (Massucci et al., 1992; Sherwood and Whittier, 1988). Therefore, it may be possible to extrapolate from studies performed in avian species. Millam and Finney (1994) found that leuprolide acetate (Lupron Depot) administered intramuscularly (reversibly) delayed egg-laying in cockatiels (*Nymphicus hollandicus*) by 12-19 days (higher doses having a longer lasting effect), compared to animals that did not receive treatment. In that study, the treatment was expected to last 28 days; but the shorter interval could have been due to the low doses and volumes used, and/or higher body temperature of birds (which would quicken metabolism of the drug) (Millam and Finney, 1994). In contrast, a study by Constantini et al. (2009) showed that a single subcutaneous administration of GnRH analogue, buserelin acetate increased laying rates and fecal estradiol metabolites in budgerigars (*Melopsittacus undulates*) compared to control groups receiving no treatment. The disparity in results between the two studies could be related to the doses used; in the study by Constantini et al. (2009) they used a dose of 10 µg/kg, whereas Millam and Finney (1994) used doses of 17, 52, or 156 µg/kg, with the most pronounced effects observed at the higher doses. Therefore, it is possible that the low dose and volume used in budgerigars was absorbed quickly, eliciting a short-term effect, resulting in stimulation of the ovaries.

Estrogen

In a study by Kummrow et al. (2010a) they examined the use of tamoxifen (an estrogen receptor blocker) implanted intracoelomically in female veiled chameleons to assess its effectiveness as a contraceptive. Although the results indicated a systemic increase in circulating estrogen immediately following implantation, no effect on the development of ovarian follicles and oviposition was observed, and additionally, in several of the animals the tablets were not completely dissolved after a period of 12-18 months. In contrast, DeNardo and Helmenski (2001) implanted leopard geckos; *E. macularius* intracoelomically with tamoxifen,

which resulted in the temporary prevention of follicular development and oviposition for one entire breeding season. The disparity in the results of these two studies may be the due to the fact that the delivery mode was ineffective in female veiled chameleons, and that could have been the cause of contraceptive failure, rather than the drug itself.

VEILED CHAMELEON (*CHAMAELEO CALYPTRATUS*)

Species Information

The veiled chameleon (*C. calyptratus*) is an arboreal lizard, native to Yemen and Saudi Arabia (Andrews and Donoghue, 2004; Kelso and Verell, 2002; Krysko et al., 2004). Captive specimens can live up to 10 years, with males living a mean of 5 years, and females 3 years (Kelso and Verell, 2002; Krysko et al., 2004). Sexual dimorphism is very apparent in this species; males can reach over 60 cm in total length, possess an enlarged cephalic casque (up to 80 mm higher than adult females), a tarsul spur on the hind foot, and hemipenal bulge at the base of the tail. Females are much shorter (35-45 cm total length) and heavier, and their casques are reduced in size. Additionally, there are distinct colour pattern difference between the two sexes; males possess a pale green or beige background, with distinct thick vertical yellow bars on the sides of the body, and dark markings along the front of the casque, around the eyes, and running horizontally along the body, whereas females exhibit an olive green background with orange markings, blue spots along the dorsal spine and casque, and often have a light-coloured lateral stripe along the length of the body.

Intraspecies communication occurs via a low-frequency buzzing and visual signals, including deliberate body movements, head jerking, and colour pattern changes (Kelso and Verell, 2002; Krysko et al., 2004). Females will adjust their behaviour and colour with reproductive state; that is whether they are receptive to courtship and mating by a male. A female that is receptive will maintain a light green background, and are not aggressive when approached by a male (although they may walk away). In contrast, females that are non-receptive will act aggressively towards males; including gaping, hissing and rocking back and forth, and will alter their colouration to a dark, near black background, and the orange and blue spots will appear much brighter. Similar (behavioural and colour change) responses have also been observed in other lizard species, including common chameleons; *Chamaeleo chameleon*

(Cuadrado & Loman, 1999; Cuadrado, 2000), Lake Eyre dragon lizards; *Ctenophorus maculosus* (Jessop et al., 2009), anole lizards (*A. carolinensis*) (McNicol and Crews, 1979), and keeled earless lizards; *Holbrookia propinqua* (Cooper and Crews, 1988; Cooper, 1984).

A number of studies have been conducted using the veiled chameleon as a model species, appearing in the following topics: ecology (Krysko et al., 2004), colour and behavioural responses to courtship (Kelso and Verell, 2002), embryonic development (Andrews and Donoghue, 2004), and reproductive endocrinology (Kummrow et al., 2010a,c,d). They serve as a good model species because of their small size, distinct colour pattern changes, early onset of sexual maturity (~5 months of age), easy maintenance, and they are readily available in the pet trade.

Reproduction

All material discussed in the following section references Kummrow et al (2010c); a study in which they characterized the reproductive hormones and morphological changes in eggs at different stages of the reproductive cycle of female veiled chameleons; unless otherwise stated.

Veiled chameleons are an oviparous (egg-bearing) species, with an average cycle length of approximately 4 months (range 112-152, average of 132.5 days). Previtellogenesis, takes up approximately two months of the reproductive cycle (range 40-80, average 52.6 days). During this stage, small primordial follicles measuring <2mm can be found on the ovaries, and the reproductive hormones (and/or metabolites), estrogen, progesterone, and testosterone are at basal levels. Estrogen production increases during vitellogenesis, and peaks in mid-late vitellogenesis, then begins to fall as progesterone and testosterone levels begin to rise. It is this decreasing estrogen:progesterone ratio which is proposed to be the trigger for ovulation. Following ovulation, eggs can be found along the length of the oviduct for approximately 2-4 weeks (8-32 days), and the sites of follicular rupture undergo rapid changes to become corpora lutea and begin to secrete progesterone. Progesterone levels peak around mid-gravidity, and then begin to decline, returning to basal levels prior to oviposition. It is hypothesized that elevated levels of progesterone during the gravid state plays a crucial role in keeping the uterus quiescent in order to prevent early expulsion of eggs prior to proper calcification.

An additional study conducted by Kummrow et al. (2010d) examined reproductive hormone patterns during non-novulatory cycles; since this species is highly prone to follicular stasis. They observed regular, cyclical hormone patterns in all animals, although the notable difference was in peak levels of fecal progesterone metabolites (pregnane) around the time that ovulation should have occurred. Peak levels of pregnane were significantly lower in non-novulatory compared to ovulatory cycles in the first and second hormone complexes (the first complex occurring at the onset of sexual maturity/colour change), but not the third; although large differences were observed when mean peak values were compared graphically.

The relationship of the HPA and HPG axes in this species is yet to be determined. Whether corticosterone plays a facilitative or inhibitory role in the reproductive cycle of female veiled chameleons has not been determined.

ASSESSMENT OF REPRODUCTIVE STATUS

Fecal hormone metabolite extraction

The use of fecal hormone metabolite extraction has become a popular method of measuring steroid concentrations of reproductive and adrenal hormones in wildlife species (Palme et al., 2005). It is a non-invasive technique which allows daily, repeated sampling from individual animals without causing capture-induced stress (Millspaugh, and Washburn, 2004; Palme et al., 2005; Touma et al., 2004). This is particularly useful for species that cannot be handled on a regular basis for blood samples, and studies which require long-term sampling. In addition, hormones are incorporated over time, are less episodic, and therefore provide a better assessment of average circulating hormone levels. This technique has been applied in a variety of mammalian and non-mammalian species, including, but not limited to: mice (Touma et al., 2005), sheep (Palme and Mostl, 1996), female veiled chameleons (Kummrow, et al., 2010a,b,c,d,), box turtles (Rittenhouse, 2005) and owls (Wasser et al., 2005).

It is hypothesized that an animals' sex, age and reproductive status may influence its adrenocortical activity, and subsequently, the circulating levels of glucocorticoids (Millspaugh, and Washburn, 2004). Therefore knowledge of these factors will aid in the accurate comparison

within and between individuals.

Imaging: Ultrasonography, magnetic resonance imaging, and radiography

Ultrasonography, radiography, and magnetic resonance imaging (MRI) are frequently used to get an “inside look” and provide immediate feedback as to the reproductive status of an animal. In a study by Kummrow et al. (2010c), they performed bi-weekly MRI scans on female veiled chameleons over the course of a normal reproductive cycle, in order to compare morphological changes in eggs to reproductive hormone activity. One important distinguishing feature they observed between pre- and post-ovulatory eggs was a distinct morphological change from appearing round and clumped together to oval, and appearing “lined up” with a thin calcified shell which became more evident closer to oviposition. They demonstrated that this method can be useful in determining the stage of the reproductive cycle when compared to reproductive hormone profiles.

Radiography and ultrasonography are commonly used to assess many chelonian and lizard species (Martinez-Torres et al., 2006; Schumacher and Toal, 2001; Sykes, 2010). Backues and Ramsay (1994) evaluated reproductive status in 3 common iguanas using radiographs and ultrasounds; they describe the appearance of follicles as round, soft tissue-opacity masses, which resembled grape clusters on radiographs; and spherical, hypoechoic, and uniform in shape on ultrasound.

There are a couple of important factors to consider when choosing which method to employ. All three methods require that the animals remain still during the examination for varying degrees of time; and thus could require the use of physical restraints or anaesthesia depending on species and individual temperament. There is a delay between capturing an image and viewing it in the case of radiography and MRI, so if more than one image needs to be taken (i.e. it the image is blurry or distorted somehow) this could require the animal to be restrained for a longer period of time. In addition, there is concern that repeated use of radiography could cause damage to germline and/or embryos, and could lead to decreased fecundity or long-term genetic problems (Sykes, 2010). Therefore this method would not be appropriate for repeated use over a long period of time. MRIs provide an excellent picture of the entire abdominal cavity and it is possible to view (and count) all of the eggs present by viewing

different planes. However, it takes a considerable amount of time to capture this data, and this technology is not readily available and/or practical for everyday use. Ultrasounds continuously generate images so it is possible to view the structures as you are passing over them. However, it is difficult to get an overall picture of the entire coelemic cavity at one time, so counting individual eggs would be extremely challenging in animals with large clutches.

Additional techniques

Additional methods that have been employed to assess reproductive status in live animals include palpation (Amey and Whittier, 2000; Moore et al., 1985; Weiss et al., 2002), transillumination (Rhen et al., 2000), and laparoscopy (Cree et al., 1990; Wibbels et al., 1992). These techniques vary in invasiveness and require training and care to perform to avoid the risk of hurting the animal. Finally, necropsy following death of an animal will reveal which structures are present in the tract (Callard et al., 1978; McPherson et al., 1982; Radder et al., 2001).

RATIONALE FOR PROJECT

A high incidence of mortality or loss of reproductive capacity in valuable breeding females can be attributed to reproductive disorders commonly encountered in captivity. The most commonly observed reproductive disorder is known as egg-binding; the retention of follicles or eggs within the reproductive tract beyond the length of a normal cycle. Little information is available describing the etiology, and prevention and treatment options of this condition. This sparked interest in developing methods to prevent its occurrence; primarily by means of contraception.

This project was intended to follow-up and expand on research carried out by Dr. Maya Kummrow, as part of her DVSc project on the endocrinology of the reproductive cycle in female veiled chameleons (*Chamaeleo calyptratus*). In the previous study, tamoxifen (an estrogen inhibitor) was evaluated as a method of contraception in this species, but determined to be unsuccessful due to a lack of absorption of the drug. The drug was intended to be administered via slow-release from a condensed pellet implanted in the coelemic cavity of the animals. However, it was later determined that the drug was not absorbed due to an immune reaction walling-off the pellets. Therefore, in the current study we wanted to examine a different route of administration, and explore the use of human-based contraceptives that are readily available.

In order to ensure all of the reproductive hormones had returned to baseline levels and vitellogenesis of the next batch of follicles had not yet commenced (based on knowledge from the previous study), the initial study design required that the animals laid eggs prior to receiving contraceptive treatment. However, only one animal successfully laid her first clutch, and concern developed over the reproductive status and health of the remaining animals that showed evidence of egg production. A high incidence of egg-binding has been reported in female veiled chameleons, so it was speculated that this was occurring in the animals that failed to lay eggs. Since the requirements of the initial study design were not met, a new objective was developed to determine the cause. This entailed employing different methods for determining the reproductive status of the animals in real-time (since logistics prevented running EIA of reproductive hormones in real-time). Once follicular stasis was confirmed in these animals, there was interest in exploring different treatment options for resolving this condition.

During the establishment of the colony, the animals had to undergo a move within the facility they were being housed in, and this resulted in a dramatic change in the external environment. It was speculated that this event triggered a stress-response and this was related to the high incidence of reproductive failure. The previous study by Dr. Kummrow did not examine the relationship between the adrenal and reproductive axes in this species, so this became an additional point of interest in the current study.

The hypothesis for this study is that follicular stasis was occurring in this population of animals, that it occurred as a result of a stress-response induced by a dramatic change to the external environment, and that this condition could be overcome using non-surgical methods. This hypothesis was addressed by the following objectives:

Objective 1: Assess the reproductive status of female veiled chameleons using hormonal, behavioural and physical techniques.

Objective 2: Examine the relationship between the adrenal and reproductive axes during normal and abnormal reproductive cycles.

Objective 3: Investigate methods to overcome follicular stasis.

The veiled chameleon was chosen as a model species for this project for a number of reasons. The primary reason was to follow up on previous work performed at the Toronto Zoo in which they characterized and validated reproductive hormone patterns associated with normal and abnormal cycles in this species. In addition, they are easy to obtain because they are bred in high numbers as part of the pet trade, their husbandry conditions are well-described, and females of this species reach sexual maturity at a young age (<6 months) which is ideal for a short term study.

The following chapters address the objectives described above. Chapter 1 of this thesis describes methods used to assess the reproductive status of the females in this study; including ultrasound imaging, male introduction trials, and fecal hormone metabolite analysis by EIA. Chapter 2 describes the relationship between the adrenal and reproductive hormone axes, and the possible response of each to the dramatic change in external environment. Finally, chapter 3 describes methods employed in attempts to overcome/resolve follicular stasis.

CHAPTER ONE

Assessment of the reproductive status of female veiled chameleons (*Chamaeleo calyptratus*) using hormonal, behavioural and physical traits

INTRODUCTION

The reproductive cycle of oviparous (egg-laying) reptiles consists of five stages: 1) previtellogenesis (PV), when primordial follicles can be found on the ovaries; 2) vitellogenesis (V), the period of rapid follicular growth and yolk deposition; 3) ovulation, expulsion of follicles from the ovary; 4) gravidity (G), when eggs are found in the oviducts; and 5) oviposition, the laying of eggs marks the end of the cycle (Kummrow et al., 2010c,d; Moore and Crews, 1986; Rhen et al., 2000). In the absence of ovulation the follicles become atreric (A) and get reabsorbed.

In captive environments animals are maintained under fairly constant husbandry conditions. They do not face seasonal alterations in temperature, humidity, prey availability, and photoperiod as they would in the wild. As a result, female reptiles in captivity produce eggs year-round, and in many species this occurs even in the absence of a male and breeding (DeNardo et al., 2002; Kummrow et al., 2010c,d; Rivera, 2008). In addition, they produce larger clutches with larger eggs compared to wildlife counterparts. This can put increased strain on the females' bodily reserves, and the potential of developing reproductive complications (Cuadrado et al., 2002; Rivera, 2008; Sykes, 2010).

Reproductive disorders are a common cause of death in captive female reptiles (Cuadrado et al., 2002; Rivera, 2008; Sykes, 2010). The most commonly observed condition, known as egg binding refers to the failure to lay eggs at the expected time based on cycle or breeding history. (Cuadrado, 2002; Kummrow et al., 2010d; Rivera, 2008; Sykes, 2010). Egg-binding can be further classified based on the location of retention within the reproductive tract; on the ovary, failure to ovulate and reabsorb yolk from atretic follicles is known as *follicular stasis*; and within the oviducts, lack of oviposition of fully formed eggs is known as *dystocia*. Although dystocia can be readily treated (and often resolved) with the administration of arginine vasotocin (AVT) or oxytocin to induce uterine contractions, and subsequently oviposition, treatment of follicular stasis is limited to individual follicle aspiration; a perilous procedure which carries a high risk of follicle rupture, and fails to prevent future occurrences (Rivera, 2008), or removal of part or all of the reproductive tract (Sykes, 2010). Retained eggs can cause inflammation, fluid build-up, compaction of the digestive tract, egg yolk coelomitis if a follicle or egg were to rupture, and, if left untreated, can result in death of the animal (Castle,1990; Rivera, 2008; Schumacher and Toal, 2001; Stacy et al., 2008). Therefore, early diagnosis of egg

binding and its location within the reproductive tract is critical to developing a treatment plan and preventing associated pathologies.

Kelso and Verrell (2002) state that female veiled chameleons (*Chamaeleo calyptratus*) can be divided into two reproductive phases; receptive or non-receptive; based on body colouration and behavioural responses when exposed (visually) to a conspecific. A female that is receptive will maintain a light green background, and will stand (or slowly walk away) when approached by a male. In contrast, females that are non-receptive will act aggressively towards males by gaping, hissing and rocking back and forth, and they will alter their colouration to a dark, near black background, and the orange and blue spots will appear much brighter (Kelso and Verell, 2002). This form of sexual signalling has also been observed in the common chameleon; *C. chameleon* (Cuadrado & Loman, 1999; Cuadrado, 2000), Lake Eyre dragon lizards; *Ctenophorus maculosus* (Jessop et al., 2009), anole lizards; *Anolis carolinensis* (Mcnicol and Crews, 1979), and keeled earless lizards; *Holbrookia propinqua* (Cooper and Crews, 1988; Cooper, 1984).

In several species of mammals, females enter a period of “estrus” or “heat” where they display behaviours which indicate their willingness/desire to mate (McNicol and Crews, 1979; Whittier and Crews, 1985). This usually occurs just prior to ovulation and is mediated by high circulating levels of estrogen produced by mature, pre-ovulatory follicles (McNicol and Crews, 1979; Whittier and Crews, 1985). Rhen et al. (2000) determined that receptive behaviour in female leopard geckos; *Eublepharis macularius* was strongly associated with circulating levels of testosterone and estradiol; hormone levels increased from pre-vitellogenesis to late vitellogenesis as did receptivity, and following ovulation, hormones returned to baseline, and females were no longer receptive. In *A. carolinensis*, estrogen and progesterone secreted in late vitellogenesis triggers receptivity in this species, and following ovulation; when progesterone remains high and estrogen levels return to baseline; females are no longer receptive (McNicol and Crews, 1979). In female veiled chameleons, similar hormone patterns are observed during pre- and post-ovulatory phases of the reproductive cycle; estrogen increases from pre-vitellogenesis to late-vitellogenesis, and declines prior to ovulation as progesterone and testosterone levels begin to rise (Kummrow et al., 2010c). Following ovulation, estrogen returns to baseline, and progesterone remains elevated, falling just prior to oviposition. During non-ovulatory cycles, estrogen cycling remains the same, but the surge of progesterone that marks ovulation is absent (Kummrow et al., 2010d). Therefore, we hypothesized that female veiled

chameleons that are close to ovulating will be receptive, and those that are post-ovulatory/gravid or failed to ovulate will be non-receptive.

Fecal hormone metabolite analysis is a technique that has become widespread in wildlife animal studies for assessing reproductive hormone patterns (Palme et al., 2005). It is non-invasive (i.e. does not require animal handling) and allows frequent, repeated sampling over long periods of time from the same individual (Millspaugh, and Washburn, 2004; Palme et al., 2005; Touma et al., 2004). Hormones are incorporated into the feces over time so they provide a more accurate reflection of average circulating levels. This technique has been applied to a variety of mammalian and non-mammalian species, including but not limited to: mice (Touma et al., 2005), sheep (Palme and Mostle, 1996), female veiled chameleons (Kummrow, et al., 2010a,c,d), box turtles (Rittenhouse, 2005) and owls (Wasser et al., 2005).

Reproductive status can also be assessed based on physical examination of the contents of the reproductive tract (i.e. follicles and/or eggs). This can be accomplished using a variety of techniques, including radiography, ultrasonography, magnetic resonance imaging (MRI), palpation, laparoscopy, transillumination, and necropsy (Amey and Whittier, 2000; Backues and Ramsay, 1994; Kramer, 2006; Kummrow et al., 2010c,d; Schumacher and Toal, 2001; Sykes, 2010). These techniques vary in invasiveness, time requirements, and effectiveness in different species. The most frequently used methods to assess reproductive status in reptiles are radiography and ultrasonography because of their low-cost and relative ease of use (Martinez-Torres et al., 2006; Schumacher and Toal, 2001; Sykes, 2010).

The objective of this study was to examine the effectiveness of different methods, including reactions to male introductions, ultrasonography, and fecal hormone metabolite analysis to assess reproductive status in female veiled chameleons.

MATERIALS AND METHODS

All procedures involving animals were approved by the Toronto Zoo Animal Care and Research Committee. The experiments were conducted in accordance with the requirements of the Animals for Research Act of Ontario and the recommendations of the Canadian Council for Animal Care.

All chemicals were obtained from Sigma-Aldrich Canada Ltd., Oakville, ON, Canada unless otherwise stated.

Animals

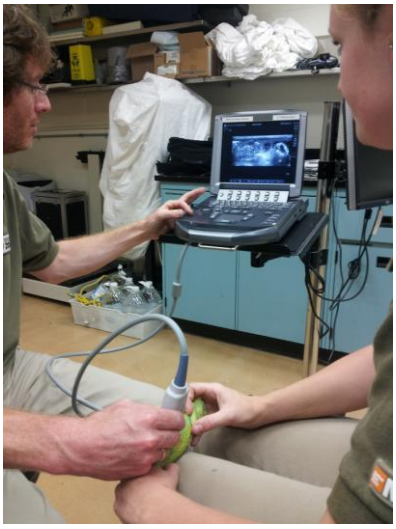
Twenty-eight female veiled chameleons born from the same clutch were acquired from a commercial reptile pet supplier in May 2012 as approximately two to three week old hatchlings. The animals were housed individually and maintained under specific husbandry conditions; including a constant 12hr light:dark cycle, with temperature and humidity maintained between 18-30°C, and 20-80%, respectively. The lighting included a F40 T12/BL/RS black light (Standard Products Inc., Saint-Laurent, QC, Canada) as UV source and a Vita-lite lamp (Duro-Test Corp., Fairfield, NJ, USA) for general lighting. The animals were fed a combination of lettuce, wax worms (*Achroia grisella*), and domestic crickets (*Acheta domestica*); dusted with the Toronto Zoo reptile supplement mix (enriched with minerals and vitamins); three days per week and misted with warm water for 1 hr twice daily. Individual animals' weights were recorded weekly for the entire study period.

Ultrasonography

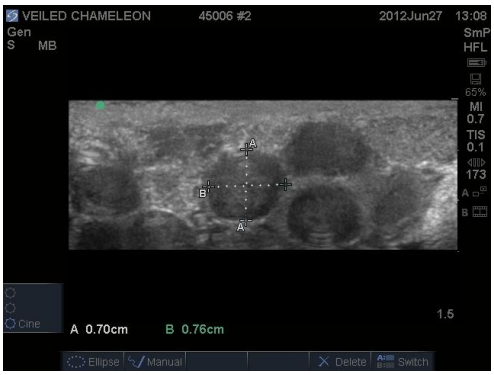
Trans-abdominal ultrasounds of the reproductive tract (and contents therein) were performed bi-weekly on all animals using a SonoSite M-Turbo ultrasound system with a SonoSite HFL38x - linear array transducer probe (Charlotte, North Carolina, United states) from February 2012 – August 2012. Animals were restrained manually in dorsal recumbence, the head and forearms held in one hand, while the other hand was used to restrain the animals' back legs while a veterinarian performed the scans (Figure 1.1a). The scans were performed in both horizontal and vertical planes in order to get a spectrum of the entire reproductive tract, to assess how "full" the animals' abdomens were, and to get the best image of the follicles or eggs within the tract. Three images were captured per individual, with the largest visible follicle (or egg) in each image measured for length, width, and surface area (Figure 1.1b,c). Based on observations from previous studies, classification of follicles and eggs was based on morphological appearance: round structures pertaining to follicles and ovoid structures with hyperechoic outline (indicative of a shell) considered eggs (Alberts, 1995; Kummrow et al., 2010c; Schumacher, 2001; Sykes, 2010).

Figure 1.1 Ultrasound procedures used to evaluate the female veiled chameleon reproductive tract

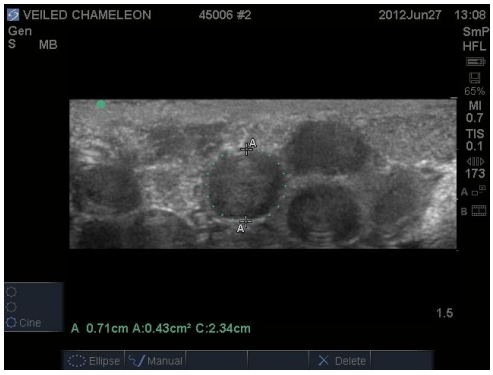
A



B



C



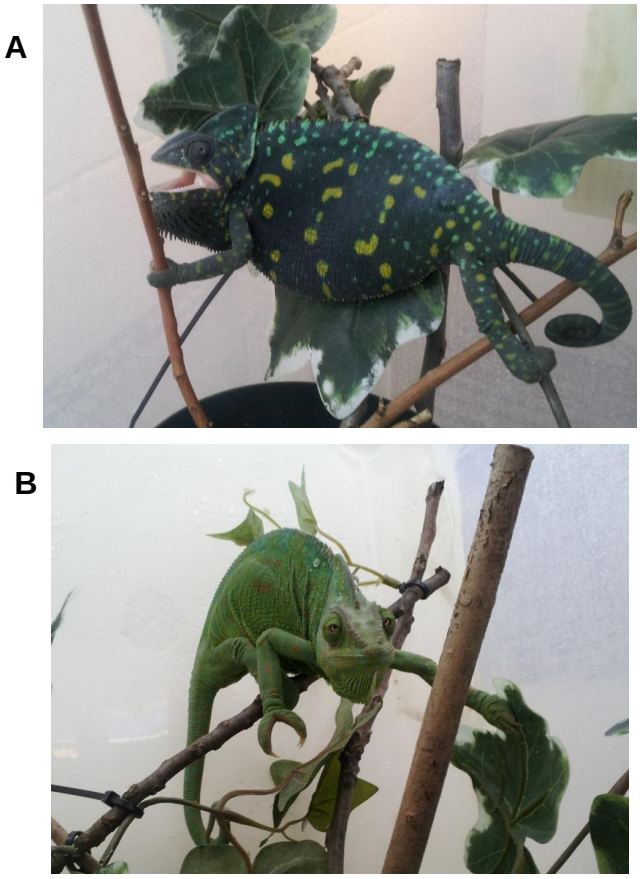
Animals were placed in dorsal recumbence, with the head and limbs restrained manually while a veterinarian scanned the reproductive tract and took measurements of follicles and/or eggs (A).

Measurements of length and width (B) and surface area (C) of follicles present in the reproductive tract of one female.

Male introduction/reaction trials

Three male veiled chameleons from the Toronto Zoo collection were used for two introduction trials, which took place 4 weeks apart. Two males were used in each trial in order to eliminate mate preference as the trigger for a reaction; i.e., the female didn't respond to one male versus another because of appearance. Subsequent trials took place on the same day, two hours apart in order to allow the females to "reset" after the first encounter (based on advice from reptile experts at the Toronto Zoo). The male was placed on a wooden stick and presented visually to each female (one at a time) by being held in front of the (mesh) cage door for up to 2 minutes; until a reaction was observed or the time ran out. Females were classified as "non-receptive" if they reacted by: 1) exhibiting a dramatic colour change, which included darkening of the background colour to near black, and the appearance of bright blue and yellow spots (Figure 1.2a); and 2) displayed aggressive behaviour towards the male which included rocking back and forth and gaping. In the majority of females that did react, this response often took place within the first 20-30 seconds of the introduction. Females that did not display this reaction (maintained a green background and showed no aggression towards males) were classified as "receptive" (Figure 1.2b).

Figure 1.2 Classification of reactions of a female veiled chameleon when visually exposed to a conspecific male



A female that is non-receptive towards a male displays a very dark green background with bright yellow and blue spots, accompanied by gaping, hissing and rocking (A). A receptive female maintains a light green background with orange and blue spots and does not display aggressive behaviour (B).

Fecal collection

Fecal samples were collected daily from all animals from October 2011-July 2012 (and continued beyond this point, up to December 2012 for 2 animals that laid eggs in July and August), prior to morning and evening misting, and within 15 hrs of defecation from all animals. The forceps used were rinsed in 80% methanol:water between collections from different animals to avoid cross contamination. Individual fecal samples were placed in labeled plastic bags (EJ

Bags, Scarborough, ON, Canada) and stored at -20°C until the extraction procedure was performed. Samples were processed for extraction within 6 months after collection and analyzed for hormone metabolites immediately thereafter.

Fecal steroid extraction

The fecal steroid extraction procedure for fecal metabolites of estrogen (estradiol; E2), progesterone (pregnane; P), and testosterone (testosterone; T) was carried out using protocols previously described by Kummrow et al. (2010b,c,d) with slight modifications. Fecal samples collected over 4 days were pooled together for analysis, then dried at 75 ºC for four hours in order to eliminate any effects of water content within the feces, after which any enclosure substrate and uric acid were scraped off. The feces were crushed and weighed out in glass scintillation vials (Fisher Scientific, Ottawa, ON, Canada) and 80% methanol:distilled water was added proportionately for 0.04 g/ml (samples weighing between 0.03 - 0.22 g). The extraction mixture was vortexed for 5-10 sec, and mixed at room temperature in a rotator at 3 rpm overnight (16-18 hrs). Samples were then centrifuged for 10 minutes at 3000 pm, after which the supernatant (fecal extract) was decanted into a second glass scintillation vial and stored at -20ºC until analysis.

Reproductive hormone enzyme immunoassays (EIA)

Fecal reproductive hormone metabolites were quantified using EIA methods previously described and validated by Kummrow et al. (2010b,c,d). Antisera (C. Munro, University of California, Davis, CA, USA) were diluted as follows: E2 (polyclonal R0008), 1:29,500; P (monoclonal CL425, Quidel Corp. San Diego, CA, USA, with final purification by C. Munro), 1:8,800; and T (polyclonal R156/7), 1:10,000. The cross-reactivities of the antisera were previously described: E2 (Walker, 1999), T (Walker, 1999), and P (Graham et al., 2001). Horseradish peroxidase conjugates (C. Munro, University of California, Davis, CA, USA) were diluted as follows: E2-HRP, 1:100,000; P-HRP 1:40,000; and T-HRP 1:20,000. Standards used were β-estradiol (Sigma E8875; 39 pg/ml – 10,000 pg/ml), testosterone (Steraloids Inc., Newport, RI, USA; A6950; 48 pg/ml – 12,500 pg/ml), and progesterone (Sigma P0130; 15.6 pg/ml – 4000 pg/ml). Controls consisted of laboratory stocks of pooled fecal extracts obtained from cycling females (E2, P) and males (T) from a variety of species that were run at 30% and

70% binding on the standard curve. Fecal extracts were diluted in EIA buffer (0.1 mM sodium phosphate buffer, pH 7.0, containing 9 g of NaCl and 1 g of BSA per litre) for hormone analysis as follows: estrogen 31-fold, testosterone 21-fold, and progesterone 10- to 410-fold.

In brief, microtitre plates (Nunc Maxisorp, VWR, Mississauga, ON, Canada) were coated with 50 µl of antiserum diluted in coating buffer (50 mM bicarbonate buffer, pH 9.6) and incubated overnight at 4 ºC. Unbound antiserum was washed from coated plates with 0.02% Tween 20 solution using a Bio-Tek ELx 405VR microplate washer (Bio-Tek Instruments, Winooski, VT, USA). Immediately following, 50 µl of fecal extracts, standards, and controls diluted in EIA buffer were added in duplicates, followed by 50 µl of horseradish peroxidase conjugate diluted in assay buffer. Plates were incubated for 2 hours at room temperature, then washed, and 100 µl of substrate solution (50 mM citrate, 1.6 mM hydrogen peroxide, and 0.4 mM 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt, pH 4.0; (Munro et al., 1991) was added. Absorbance was measured at 405 nm using a spectrophotometer (MRX microplate reader, Dynex Technologies, Chantilly, VA, USA). Fecal hormone levels are presented as mass/g dry weight.

Statistical analysis

Statistical analyses were performed using SAS 9.1.2 (SAS Institute Inc., Cary, NC, USA) and Excel (Microsoft Office Excel 2007, Microsoft, Redmond, WA, USA).

Baseline values were calculated for each hormone from all individuals from fecal samples collected from October 2011 – July 2012; using methods described by Kummrow et al. (2010c,d). In brief, an iterative process was used in which values exceeding the mean plus 1.5 standard deviations (SD) were removed. The mean was then re-calculated and the elimination process repeated until no values exceeded the mean plus 1.5 SD. The final calculated mean was considered the baseline value. A cluster of two or more values above baseline + 1.5 SD was considered a hormone peak. The peak maximum value was calculated as the difference between the highest measurement within the hormone peak and hormone baseline value, measured in ng hormone per g dry fecal matter.

Hormone metabolite values were graphed against time (October 2011 – July 2012),

recognizing the fact that each measurement represented a sample pooled over four days and that hormones appear in the feces some days later than their presence in circulating blood (Klasing, 2005; Millspaugh and Washburn, 2004).

The difference in size and shape between follicles and eggs were measured using the following response variables: 1) the ratio of length/width, and 2) surface area, using repeated measures over time of animals that were known to have ovulated. Specifically, this included data from the last two dates prior to ovulation for follicles (assigned times 1 & 2), and the first date following ovulation for eggs (time 3). The explanatory variables were time along the maturation/transition period and calliper (not included for the surface area model). A general linear mixed model was fitted to the data, using Proc MIXED (SAS version 9.1.2). To account for the repeated measures, the following error structures were attempted: random effects with sub-sampling (i.e., variance component), AR(1), ARH(1), Toep, Toep(2), ToepH, ToepH(2), unstructured, and unstructured(2).

To assess ANOVA assumptions, residual analyses were performed, including formally testing the residuals for normality, using the four tests offered by SAS (Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises, and Anderson-Darling). The residuals were plotted against the predicted values and explanatory variables used in the model (caliper, ID). Such analyses may reveal the need for transformations, outliers or other factors to consider.

Biological data

Dates of oviposition were recorded, and necropsies were performed at the end of the study, at which time the reproductive tract was removed and evaluated. Two follicles and/or eggs from each ovary/oviduct were measured for length and width using electronic calipers (carbon-fiber composite digital caliper 6 inches; Traceable Calibration Control Company, Friendswood, Texas, United Sates; Fisher Scientific cat#15-077-958), and notes on weight, colour and vascularity were recorded. An ultrasound of one ovary removed from the tract post-necropsy was performed by submersing it in water, in order to determine if new and old follicles would appear different on ultrasound.

RESULTS

Unexpectedly, the majority of the females in the colony became egg-bound with only 1 of the 28 females laying eggs at the expected time (and one of the females was diagnosed with the condition earlier on and was spayed as a result). The remaining animals exhibited distended abdomens, with swellings in the inguinal regions (indicative of follicle or egg presence) for a length of time which exceeded the length of a normal cycle in this species (based on data from a previous study; Kummrow, et al., 2010c).

Ultrasonography

Comparison of follicle and egg morphology

During the early examinations it was difficult to differentiate follicles from eggs on ultrasound images. The threshold for distinguishing “round” versus “ovoid” could not be defined as many of the structures observed were distorted in shape from being compacted against neighbouring structures and the outline echogenecity was not distinct. However, a distinct change in morphology indicated a transition from pre-ovulatory follicles to eggs between consecutive examinations of the same animal when ovulation would have occurred (Figure 1.3). This event was confirmed by hormone pattern analysis, and oviposition occurring within the 1-3 weeks following the first appearance of eggs on ultrasound. Thereafter it was possible to differentiate follicles from eggs when evaluating consecutive images of the same individual.

Follicles at time 1 measured on average 0.94 cm for length (range = 0.86-1.04 cm), and 0.76 cm for width (0.68-0.89 cm). At time 2 the average length was 1.01 cm (1.0-1.04 cm), and width, 0.85 cm (0.76-0.95 cm); based on measurements from 4 animals. Post-ovulatory eggs measured an average of 1.27 cm (1.03-1.46 cm) for length, and 0.80 cm (0.66-0.97 cm) for width, based on measurements from 7 animals, including those measured with calipers post-necropsy.

For the ratio response, based on the AIC, none of the error structures considered were found to be needed, resulting in a purely fixed-effects model. For the surface area, the AIC indicated a variance component model was appropriate. For both responses, residual analyses

indicated the ANOVA assumptions were adequately met. The difference between using calipers versus ultrasounds for measuring the length and width of eggs was examined, and no effect was found ($p>0.25$) and time and caliper was not significant ($p>0.5$).

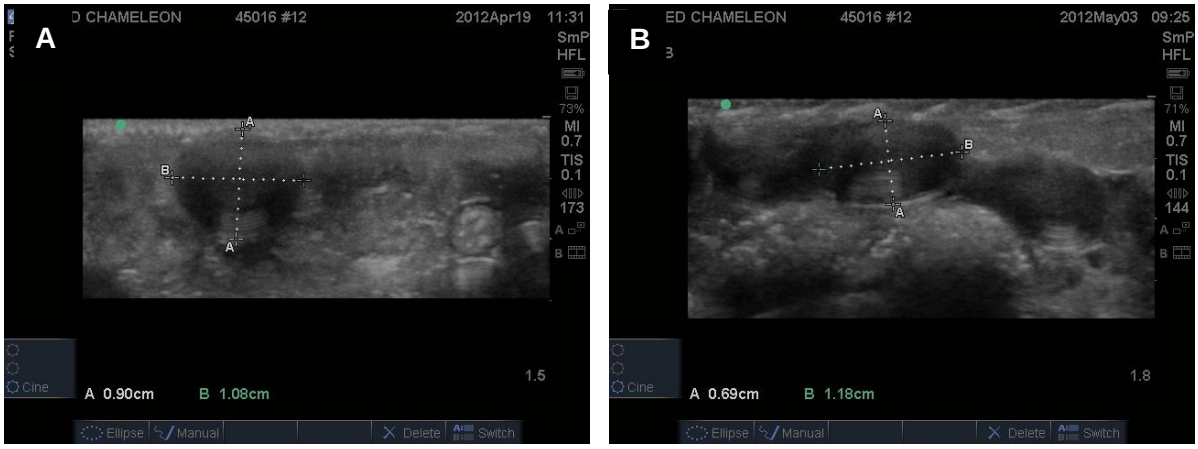
On average the length/width ratios of follicles and eggs were found to be statistically different ($p<0.0001$). No difference was found between times 1 and 2, so times 1 and 2 were averaged and the difference between that average and the ratio for time 3 was determined. The time 1 ratio was on average 1.25 (95% CI = 1.18, 1.33); time 2, 1.21 (95% CI = 1.12, 1.30); and time 3, 1.59 (95% CI = 1.52, 1.67), and averaging over times 1 and 2, the ratio is estimated to be 1.23 (95% CI = 1.17, 1.29) (Table 1.1). Therefore the average difference between time 3 and the average of times 1 and 2 is 0.37 ($p<0.0001$; 95% CI = 0.27, 0.46).

Based on the 1-sided 90% tolerance intervals, the following rules apply to the current population: 1) in the animals that had eggs, 95% of such individuals had ratios larger than 1.27, hence if the ratio is less than that, it is fairly unlikely to be an egg; 2) in animals that did not have eggs, 95% of follicles measured were less than 1.56, hence if the ratio exceeds 1.56, it is fairly likely to be an egg (even though some follicles produce ratios higher than that); and 3) a grey zone exists between 1.27 and 1.56, were it is not possible to distinguish a follicle from an egg.

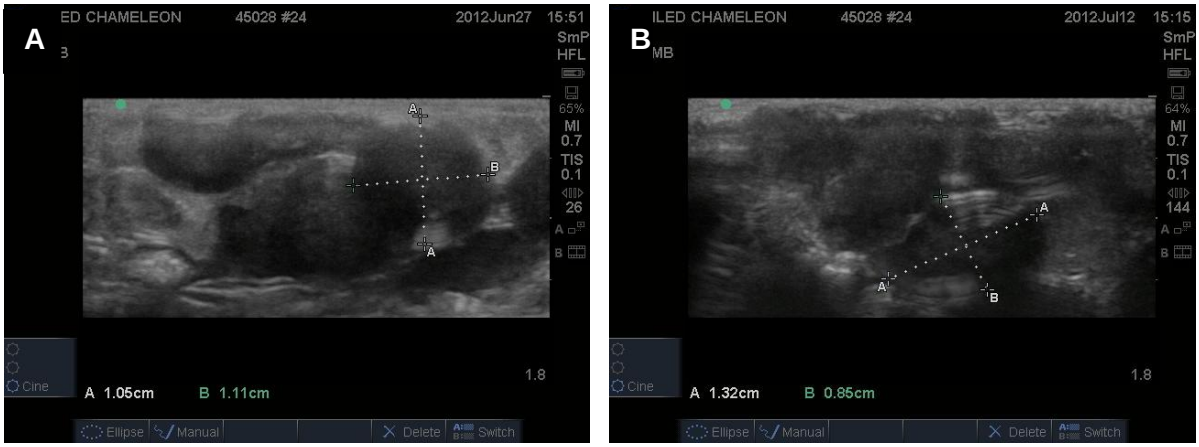
For the surface areas, all three means differed. The mean for time 1 was 0.58 cm^2 (95% CI = 0.53, 0.63 cm^2); time 2, 0.69 cm^2 (95% CI =0.63, 0.75 cm^2); and time 3, 0.83 cm^2 (95% CI =0.76, 0.90 cm^2) (Table 1.1). The difference in means for time 2 and time 1 was 0.11 cm^2 ($p=0.0003$, CI = 0.05, 0.16 cm^2) time 3 and time 1, 0.25 cm^2 ($p<0.0001$, CI = 0.19, 0.31 cm^2); and time 3 and time 2, 0.14 cm^2 ($p<0.0001$, CI = 0.08, 0.21 cm^2). In order to determine if the rate of change was different between times 2 and 3 than it was between times 1 and 2, a contrast was constructed to test this idea. There was no difference found ($p=0.50$), which suggested that the rate of growth was constant.

Figure 1.3 Ultrasound images of pre-ovulatory follicles and eggs in the reproductive tract of female veiled chameleons

45016



45028



Ultrasound images of pre-ovulatory follicles (A) and eggs (B) from two female veiled chameleons (45016 and 45028) that ovulated during the study period.

Table 1.1 Measurements of follicles and eggs taken from ultrasound images of the reproductive tract of female veiled chameleons

Time	Length (cm)	Width (cm)	Length:Width	Surface Area (cm^2)
1	0.94 (0.86-1.04)	0.76 (0.68-0.89)	1.25 (1.18, 1.33)	0.58 (0.53, 0.63)
2	1.01 (1.0-1.04)	0.85 (0.76-0.95)	1.21 (1.12, 1.30)	0.69 (0.63, 0.75)
3	1.27 (1.03-1.46)	0.80 (0.66-0.97)	1.59 (1.52, 1.67)	0.83 (0.76, 0.90)

Calculated means for length, width, length:width ratio, and surface area for follicles (time 1 &2) and eggs (time 3) along with ranges for length and width, and 95% confidence intervals for ratios and surface area (in brackets).

Confirmation of follicular stasis

During the first six weeks of ultrasound examinations, large (>8 mm in diameter), round structures, assumed to be follicles were observed within the reproductive tracts of the majority of the females. Comparison of measurements indicated that these structures had not changed significantly in size or shape between consecutive exams over several weeks. Based on these data, the affected animals were diagnosed with follicular stasis.

Reproductive hormone analysis

The stage of the reproductive cycle was estimated based on hormone profiles generated by enzyme immunoassay (EIA) of the fecal hormone metabolites (E2, P and T). Females were classified into stages based on criteria previously described by Kummrow et al. (2010c,d) as follows: animals with baseline levels of E2, T and P were considered to be in previtellogenesis (PV); the period when levels of E2 rose above baseline up to a peak, and the period immediately thereafter when levels began to fall and T and P increased was considered vitellogenesis (V); a surge of progesterone (approximately 20 fold increase over mean baseline P values) indicated ovulation had occurred, so the period immediately following, when P levels remained elevated was the gravid (G) phase, oviposition occurred immediately following a drop in P. In the absence of ovulation, E2 levels falling and returning to baseline following a peak was classified as the start of atresia (A). The vitellogenic period was further broken down into early,

mid, and late stages as these corresponded to differing levels of E2, T and P. The cycle lengths of animals in the current population could not be estimated accurately since the relevant biological event (egg-laying) that defines the start and end points of the cycles were absent in all of the animals.

Follicular changes and endocrine profiles

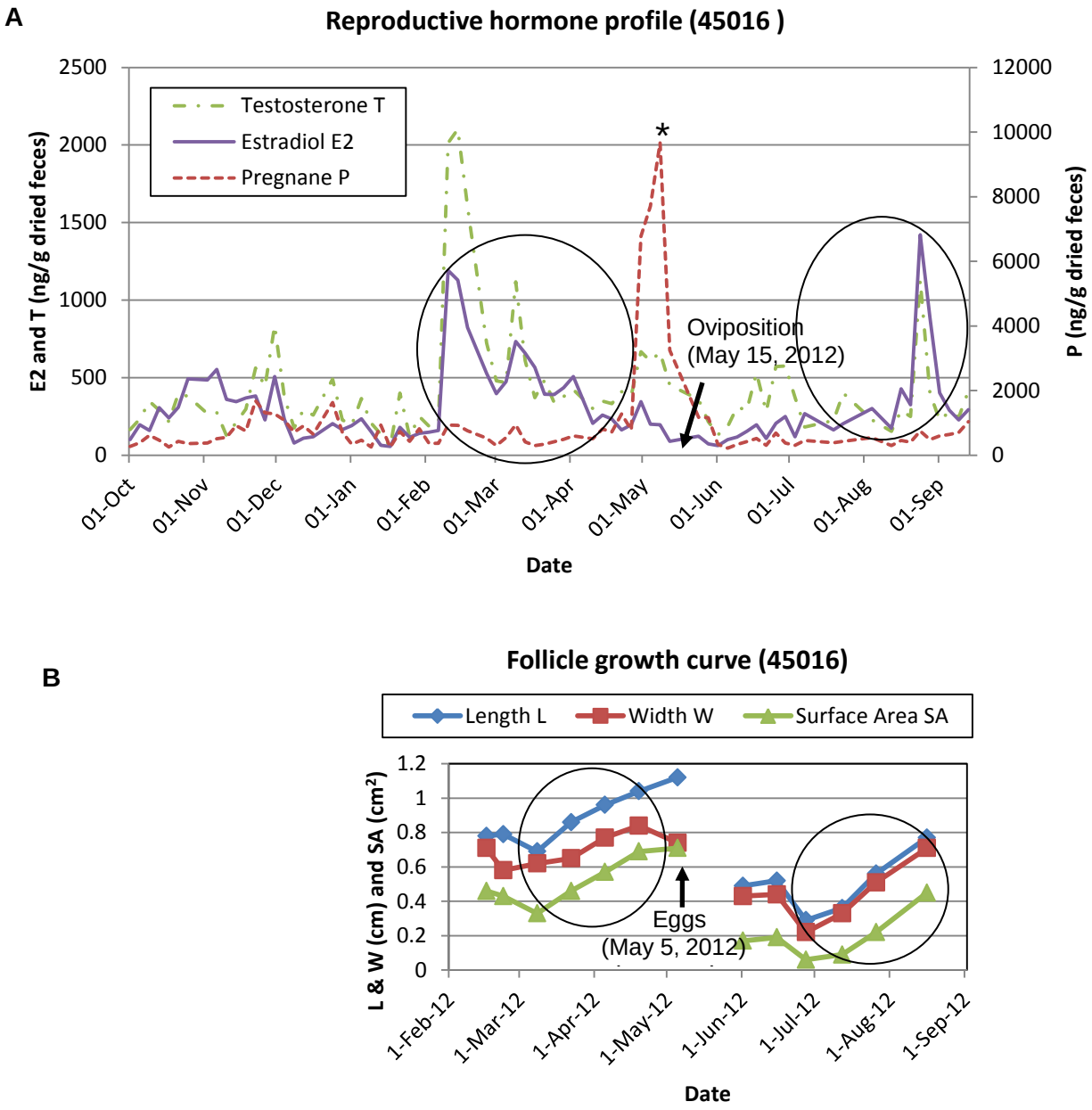
Individual hormone profiles and follicle growth curves from each chameleon is available in Supplemental Materials 1 (available in pdf format).

Periods of follicular growth/vitellogenesis corresponded with increasing levels of E2 (Figures 1.4 & 1.5). A distinct peak in pregnane (>20-fold increase above baseline) occurred within the period between the transition from follicles to eggs, and levels subsequently dropped just prior oviposition (Figure 1.4). In the absence of ovulation, follicles began to reduce in size (to a point), and E2 levels returned to baseline (Figure 1.5).

Confirmation of non-ovulatory cycles

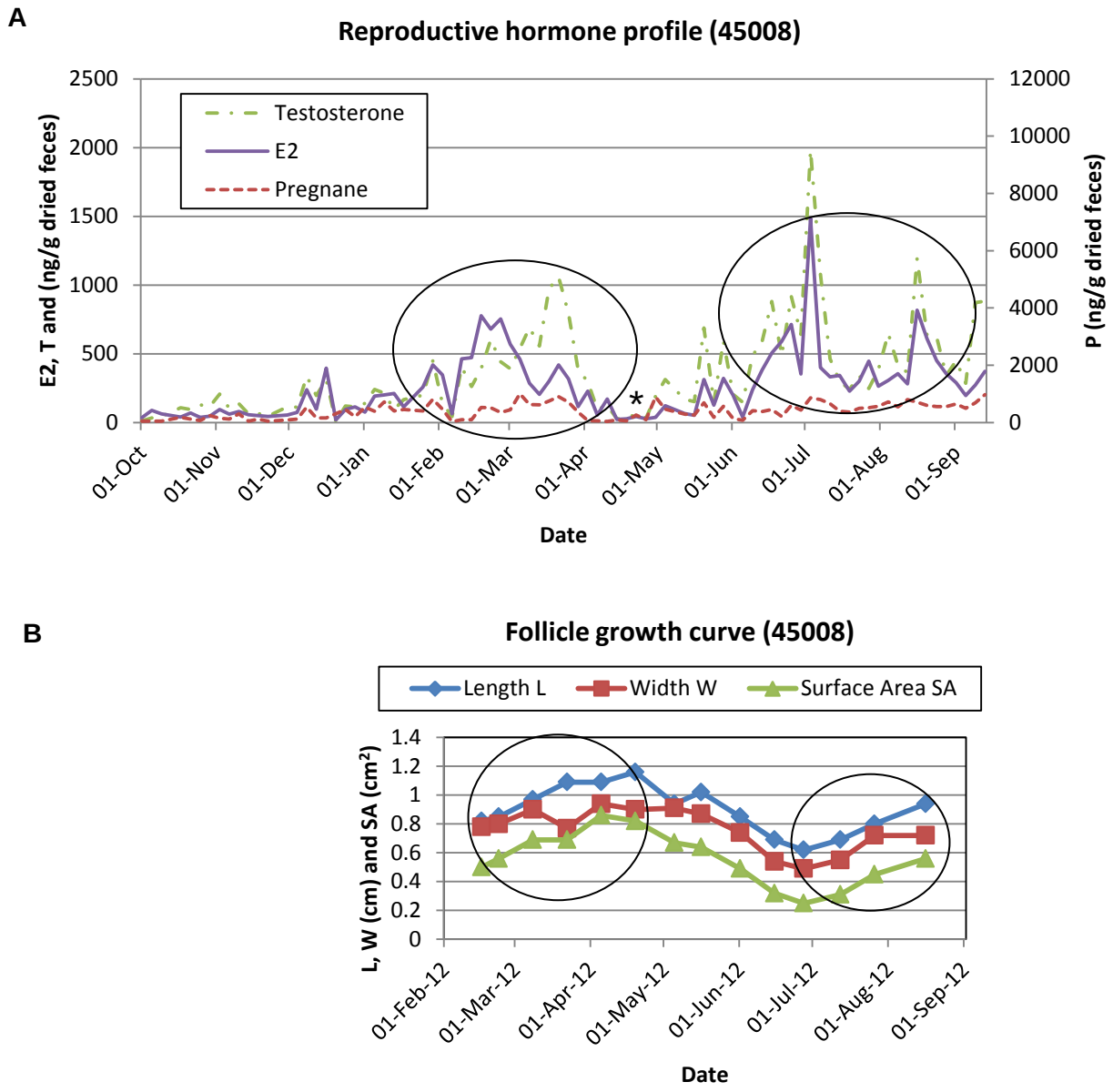
In the majority of the animals, there was evidence of vitellogenesis, marked by increasing levels of estrogen, but an absence of the surge of progesterone (evidence that ovulation occurred) at the expected time (Figure 1.5). This confirmed that the animals had produced a clutch of follicles but failed to ovulate them.

Figure 1.4 Fecal hormone patterns and follicle growth patterns associated with an ovulatory cycle in a female veiled chameleon



Reproductive hormone profile of an animal that laid eggs (A) with periods of vitellogenic growth circled, post-ovulatory surge in P indicated by an asterix, and date of oviposition indicated. Follicle growth curve from the same animal (B) with periods of vitellogenic growth circled, and date eggs first appeared on ultrasound indicated.

Figure 1.5 Fecal hormone patterns and follicle growth patterns associated with a non-ovulatory cycle in a female veiled chameleon.



Reproductive hormone patterns (A) of an animal that did not ovulate with periods of vitellogenic growth circled, and timing where post-ovulatory surge in progesterone should have occurred indicated by an asterix. Follicle growth curve from the same animal (B) with periods of vitellogenic growth circled. Fecal hormone metabolites measured were pregnane (P), testosterone (T), and estradiol (E2).

Male introduction/reaction trials

Results of the male introduction trial, along with the stage of the reproductive cycle the females were in at the time of the introductions are illustrated in Table 1.2. Seven of the females reacted in the first introduction trial, 6/7 of which were in PV, the 7th in mid-late V. The remaining 21 animals that did not react showed variation in reproductive stage (Table 1.2). In the second trial, of the animals that reacted, 3/6 were in PV and 3/6 in early V. The remaining 22 animals varied across all of the reproductive stages (Table 1.2).

Table 1.2 Male introduction/reaction trial results

		Cycle stage					
Trial	**Reaction**	**PV**	**Ve**	**Vm**	**Vl**	**G**	**A**
1	Yes (n=7)	6	0	0	1	0	0
	No (n=21)	10	4	1	3	1	2
2	Yes (n=6)	3	3	0	0	0	0
	No (n=22)	6	3	6	2	1	2

Cycle stage of female veiled chameleons that did or did not react upon visual exposure to a conspecific male. PV = previtellogenesis; V = vitellogenesis (e = early; m = mid; and l = late); G = gravid; A = atresia

Biological data

One female laid eggs during the expected time period for first oviposition following sexual maturity on February 3, 2012 (45023). The remaining dates of oviposition were, May 15, 2012 (45016), July 28, 2012 (45028) and August 20, 2012 (45030).

Post-Necropsy

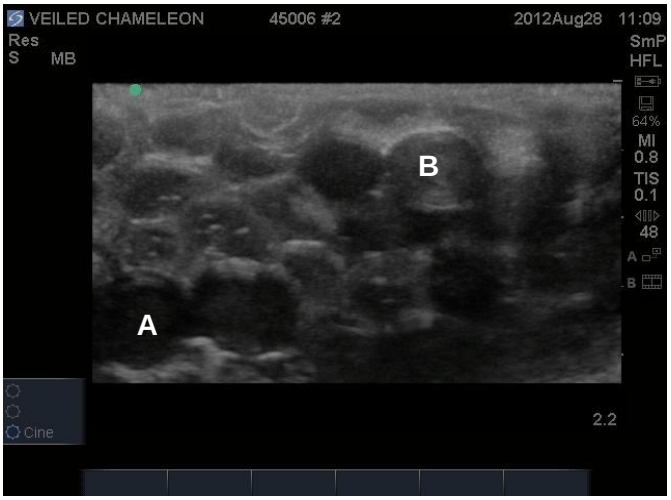
It was possible to distinguish between “new” and “old” batches of follicles on the ovaries of animals necropsied; newer batches were well vascularised, bright yellow, round, and turgid,

whereas old follicles were dark yellow, collapsible, and not well vascularised (Figure 1.6). These differences were also apparent on ultrasound images taken of an ovary post-necropsy (image is not available, Figure 1.7 displays ovaries within an animal that had both new and old follicles present in the tract).

Figure 1.6 Post-necropsy photographs of excised ovaries from female veiled chameleons

Ovaries removed from two animals following necropsy. “Old” (A) and “new” (B) batches of follicles identified; old follicles appear dark yellow, collapsible and not well vascularised, new follicles appear bright yellow, turgid, and well vascularised.

Figure 1.7 Ultrasound image containing new and old follicles on the ovaries of a female veiled chameleon



Ultrasound image of an animal taking immediately prior to necropsy; “old” (A) and “new” (B) follicles identified based on echogenecity.

DISCUSSION

Based on observations from previous studies, the initial classification of follicles and eggs was based on shape; follicles having a round appearance, and eggs appearing ovoid (Alberts, 1995; Kummrow et al, 2010c; Martinez-Torres et al., 2006; Schumacher and Toal, 2001; Sykes, 2010). Although there was a significant difference in the length/width ratios of follicles and eggs, there was a broad range of values for both follicles and eggs within and among animals in this study. In some instances follicles produced ratio values that were quite large, and close to the estimated mean ratio of eggs. The reverse also occurred; some eggs produced ratio values closer to the estimated mean values for follicles. In the current study, it was the morphological change that occurred between consecutive images on the same individuals that indicated a transition from the pre- to post-ovulatory structures. These observations demonstrate the difficulty in identifying ovarian status from one ultrasound examination. However, use of repeated, consecutive exams on the same animal over 3-4 weeks can provide accurate data to determine whether changes in size and shape are occurring, or whether the structures are remaining static.

Schumacher and Toal (2001) stated that: in reptiles, preovulatory follicles are hypoechoic, whereas postovulatory follicles are more hyperechoic, with a readily identifiable calcified shell. In the current study the echogenecity of these structures and/or their outlines was not a reliable indicator of follicles versus eggs. In fact, some follicles possessed a hyperechoic outline, and some of the eggs did not. It is possible; however, that eggs identified on ultrasound were not far along in the calcification process, or that the shell of veiled chameleon eggs is not thick enough to distinguish on ultrasound.

Observations taken of gross appearance of the reproductive tract following necropsy provided a means to distinguish between “new” and “old” batches of follicles. New batches were well vascularised, bright yellow, round, and turgid, whereas old follicles were dark yellow, collapsible, and not well vascularised. Crews (1975) made similar observations on the appearance of atretic follicles in *A. carolinensis*, although in that study the atretic follicles were

well vascularised, possibly attributed to active reabsorption taking place which was not occurring in the females in the current study. Ultrasonography of an ovary removed from the tract of an animal post-necropsy, and submersed in water indicated there was an observable difference between “new” and “old” batches of follicles. In the future this information could be applied to distinguish new and old follicles within the reproductive tract of a live animal, which would be vital for the early diagnosis and development of a treatment plan for (resolving) follicular stasis.

The results of the ultrasound examinations generated follicular growth curves, which correlated with the reproductive hormone activity, and were consistent with results obtained by Kummrow et al. (2010c), where they used MRIs and fecal hormone metabolite analyses to evaluate ovarian activity throughout the reproductive cycle of the same species. This confirms that ultrasonography is a reliable method to track changes in follicular growth over the course of the reproductive cycle, and can be used to identify animals with retained follicles or eggs if exams are performed on a consistent basis. The specific trigger for ovulation could not be confirmed in the current study because this event occurred within a two week window between subsequent ultrasound exams so it was not possible to compare the date of ovulation with corresponding hormone metabolite levels at that time. Kummrow et al. (2010c) suggest that it is the ratio of decreasing estrogen:progesterone that prepares the follicles for ovulation in this species, and they propose that this occurs as a result of a surge in gonadotropins initiating a transition in the follicular granulosa cell layers to secrete progesterone instead of estrogen. The specific mechanism of ovulation has not been determined in reptiles. In mammals, a surge in leutenizing hormone (LH) is the trigger for ovulation, but no LH-like molecule has been identified in lizards to date (Callard, 1988; Licht, 1984), although, Aizawa and Ishii (2003) determined the full-length cDNA encoding the LH β subunit precursor molecule of the Japanese grass lizard (*Takydromus tachydromoides*), and deduced the final amino acid sequence of the mature peptide. However, the expression of this gene throughout different stages of the reproductive cycle has not been studied, and the biological significance remains obscure. It is therefore proposed that beyond promoting follicle maturation, FSH may also be involved in ovulation. Therefore, further studies investigating FSH levels around the time of ovulation are warranted to determine its role in the process of ovulation.

In four out of five animals that laid eggs throughout the study period (February-August), medium-large sized follicles (>5mm) were identified in the tract immediately following

oviposition. Under normal circumstances, there is a refractory period following ovulation; when pregnane levels are elevated, and this prevents the recruitment of a new batch of follicles (Kummrow et al., 2010c). Furthermore, during previtellogenesis (which encompasses the first month of the reproductive cycle), follicles measuring <2mm in diameter can be found on the ovary following oviposition. In the current study, this was observed in the animal that successfully laid the first cohort of eggs produced (as evidenced by hormone pattern analysis). Therefore, the medium-large follicles (>5mm in diameter) present on the ovary at this time would have originated from a previous clutch that had failed to ovulate. Reproductive hormone profiles confirmed this was the case as evidenced by a non-productive cycle (estrogen wave which was not followed by a peak in progesterone) followed by a productive cycle (estrogen wave followed by a peak in progesterone, indicating ovulation had occurred).

The responses of females to male introductions in the current study were not a clear indicator of animals that were in pre- and post-ovulatory phases of the reproductive. Furthermore, female receptivity did not vary with reproductive hormone production or follicle size. In fact, the one animal that was gravid (post-ovulatory) during both trials did not react to any of the males. These findings are in contrast to results obtained by Jessop et al. (2009), McNicol and Crews (1979), and Rhen et al. (2000) where they found that female receptivity varied with the stage of the reproductive cycle in Lake Eyre dragon lizards (*C. maculosus*), anole lizards (*A. carolinensis*), and leopard geckos (*E. macularius*), respectively. In all of these studies, female receptivity increased from previtellogenic-late vitellogenic stages of ovarian growth, which was directly related to i) increased estrogen production in combination with progesterone (anole lizards), ii) testosterone and DHT (leopard geckos), and iii) increased progesterone and testosterone production (Lake Eyre dragon lizards) in late vitellogenesis. Receptivity declined substantially following ovulation, and this was associated with elevated progesterone levels throughout the gravid period (Jessop et al., 2009; McNicol and Crews, 1979; Rhen et al., 2000).

CONCLUSION

It is possible to distinguish follicles from eggs using ultrasound imaging when assessments are performed bi-weekly for the duration of a normal reproductive cycle. Although ratios of length/width do not serve as a reliable method to classify follicles versus eggs, the distinct morphological change that occurs following ovulation can serve as a good indication that

the animal is in the gravid stage of the cycle and will be laying eggs in the coming weeks. Ultrasound imaging can also be used to confirm suspicions of follicular stasis; by looking at the echogenecity, and if performed over several weeks, the lack of significant change in size and shape over time. Female receptivity to male introductions does not serve as a reliable method to identify females in the pre- or post-ovulatory stages of their reproductive cycles. Fecal hormone metabolite analysis is a reliable method to confirm whether or not ovulation occurred in an animal; but it is not always logistically possible to obtain results immediately, in particular for large numbers of animals.

CHAPTER TWO

Identifying the role of stress and adrenal hormone production during normal and abnormal reproductive cycles in female veiled chameleons (*Chamaeleo calyptratus)*

INTRODUCTION

Animals respond behaviourally and physiologically to stressors in order to conserve energy and increase their chances of survival (Millspaugh and Washburn, 2004; Moore and Jessop, 2003; Palme et al., 2005). This is often accompanied by activation of the hypothalamic-pituitary-adrenal (HPA) axis and down-regulation of the hypothalamic-pituitary-gonadal (HPG) axis and reproductive behaviours. Corticotropin releasing hormone (CRH), produced by the hypothalamus, is the first hormone in the HPA axis, and it is released when a stressor is detected. CRH travels through the portal circulation and binds to receptors on the pituitary gland, which elicits the increased production and release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH travels through the systemic circulation and binds to receptors on the adrenal glands, which causes increased production and release of glucocorticoids (GCs) from the adrenal cortex (Palme et al., 2005). In reptiles, the main glucocorticoid produced is corticosterone; in contrast to mammals which produce cortisol (Millspaugh and Washburn, 2004).

The relationship between stress and reproduction is complex, and is highly context-dependent (Moore and Jessop, 2003). Traditionally “stress” has been viewed as inhibitory to reproduction, because down-regulation of the hypothalamic-pituitary-gondal (HPG) axis and reproductive behaviours occurs as a means to conserve energy. However, this is not a universal phenomenon; as some species will endure stress (by down-regulating the HPA axis) in order to complete reproductive activities (Moore and Jessop, 2003). In addition, in some species, stress or more accurately the production of glucocorticoids, can occur as a result or bi-product of the high energy demands associated with courtship, mating and the production of gametes (Millspaugh and Washburn, 2004; Moore and Jessop, 2003). In fact, they may even have a facilitative role in reproductive processes.

In certain reptile species systemic levels of glucocorticoids are elevated throughout the breeding season compared to the non-breeding season (Greenberg and Wingfield, 1987; Millspaugh and Washburn, 2004; Moore and Jessop, 2003). This has been observed in side-blotched lizards; *Uta stansburiana* (Wilson and Wingfield, 1992), red-sided garter snakes; *Thamnophis sirtalis parietalis* (Moore et al., 2001), and eastern fence lizards, *Sceloporus undulates* (John-Alder et al., 2002). They may also vary with different stages of the reproductive cycle (i.e. previtellogenesis, vitellogenesis, and gravidity), as seen in *U. stansburia* (Wilson and

Wingfield, 1992), *Cnemidophorus uniparens* (Grassman and Crews, 1990), *Tuatara sphenodon punctatus* (Tyrell and Cree, 1998), and *Lacerta vivipara* (Dauphin-Villemant et al., 1990). However, there are also species in which plasma corticosterone levels do not appear to be elevated during the breeding season, such as bearded dragon lizards; *Pagona barbata* (Amey and Whittier, 2000), and New Zealand common geckos; *Hoplodactylus maculates* (Girling and Cree, 1995).

It is therefore important to establish data on "normal" patterns of glucocorticoid metabolite excretion in a given species, in order to determine the impact of potential confounding factors (Millspaugh and Washburn, 2004).

A high incidence of follicular stasis affecting females in the current study (described in chapter 1) prompted investigation into whether a change in the external environment elicited a stress response, and subsequently if this had a negative impact on reproductive activity.

The objectives of this study were to evaluate the relationship between adrenal and reproductive hormone output, by means of enzyme immunoassay (EIA) to detect levels of fecal hormone metabolites; pregnane (P), estradiol (E2), testosterone (T), and corticosterone (CORT) in female veiled chameleons (*Chamaeleo calyptratus*) and determine the impact of changes to the external environmental on both axes.

MATERIALS AND METHODS

The animals used in this portion of the study were the same individuals from the colony discussed in chapter 1. Detailed information on husbandry conditions and methods used for fecal collection, hormone metabolite extraction, EIA of fecal reproductive hormones, and ultrasound images are described therein.

During establishment of the research colony, animal management requirements resulted in the animals to undergoing a change in the external environment. They were moved to a new a room with a different layout, into a holding pen that was surrounded by enclosures containing other animals (they had been the sole occupiers of the previous room).

Fecal extraction efficiency (corticosterone)

Efficiency of the extraction procedure was analyzed through recovery of exogenous corticosterone added to the fecal samples before extraction. Five pooled fecal samples (0.2g each) were each spiked with either corticosterone standard or no hormone. The samples were mixed and extracted as described previously. The percent efficiency was calculated using the following formula: amount observed/amount expected x 100%; where amount observed is the value obtained from the spiked sample minus background and amount expected is the calculated amount of corticosterone standard added. The percent efficiency is presented as mean ± standard error of the mean (SEM).

Enzyme immunoassays (EIA)

EIAs for reproductive hormone metabolites: E2, P and T were run as described in chapter 1.

Fecal corticosterone metabolites (corticosterone; CORT) were quantified using EIA methods previously described (Watson et al., 2013; Metrione and Harder, 2011). Antisera were diluted as follows: goat anti-rabbit IgG (GARG) polyclonal antibody (Sigma-Aldrich, Canada), 25 µg/well; and CORT (polyclonal CJM006, C. Munro, University of California, Davis, CA, USA) was diluted 1:200,000. The cross-reactivities of the antisera were previously described: GARG and CORT (Watson et al., 2013; Metrione and Harder, 2011). CORT horseradish peroxidase (HRP) conjugate (C. Munro, University of California, Davis, CA, USA) was diluted 1:1,000,000. Standards used were corticosterone (Steraloids Q1550; 39 pg/ml – 10,000 pg/ml). Controls consisted of laboratory stocks of pooled fecal extracts obtained from female spotted-necked otters (*Hydrictis maculicollis*) that were run at 25% and 65% binding. Fecal extracts were diluted in EIA buffer (0.1 mM sodium phosphate buffer, pH 7.0, containing 9 g of NaCl and 1 g of BSA per litre;) for hormone analysis 10-fold.

In brief, microtitre plates (Nunc Maxisorp, VWR, Mississauga, ON, Canada) were coated with goat anti-rabbit IgG polyclonal antibody (Sigma-Aldrich Canada) 0.25 µg/well diluted in coating buffer (50mM bicarbonate buffer, pH 9.6). After overnight incubation at room temperature in the dark, plates were washed with 0.05% Tween 20, 0.15 M NaCl solution using a Bio-Tek ELx 405VR microplate washer (Bio-Tek Instruments, Winooski, VT) and blocked with

250 µl EIA buffer per well for minimum 1 h at room temperature. Soon after, 50 µl of fecal extracts, standards, and controls diluted in EIA buffer were added in duplicates followed by 100 µl of horseradish peroxidase conjugate and 100 µl CORT antiserum diluted in EIA buffer. Plates were incubated overnight in the dark at room temperature. On the third day, plates were washed and 200 µl of substrate solution (0.5 ml of 4 mg/ml tetramethylbenzidine in dimethylsulphoxide and 0.1 ml of 0.176 M H_2O_2 diluted in 22 ml of 0.01 M $C_2H_3NaO_2 \cdot 3H_2O$, pH 5.0) was added. After 30 min in the dark at room temperature, the colour development was stopped with 50 µl H_2SO_4 (1.8 M). Absorbance was measured at 450 nm using a spectrophotometer (MRX microplate reader, Dynex Technologies, Chantilly, VA).

CORT EIA validation

Parallelism

Parallel displacement between the standard curve and serial dilutions of fecal extract was used as an indirect measure of assay specificity. Parallelism indicated immunological similarities between the standard and sample hormone levels. A representative pooled sample of fecal extracts was serially diluted two-fold between 1:2 to 1:512 in assay buffer and run on the CORT assay alongside the standard curve. The graph was plotted as relative dose vs. percent antibody bound and linear regression analysis of the resulting curves was performed. Sample dilution was selected based on 50% binding of the pooled sample curve.

Precision

To assess repeatability of results, calculation of intra- and inter-assay coefficients of variation (CV's) was performed. Intra-assay CV's were consistently monitored on each plate in real time by examining the CV of each duplicate run on the plate. Only values from duplicates with <10% CV were recorded as data. Intra-assay CV's were further evaluated using a pooled extract at 50% binding loaded in different spots on the plate, and this was repeated three times. Inter-assay CV's were evaluated using fecal extract controls (25% and 65% binding) loaded in duplicate on each plate.

Accuracy

To examine possible interference of components within the extract with antibody binding, recovery of a known amount of corticosterone was calculated. A pooled sample of fecal extracts diluted to the usual range for unknown samples was used. To 100 µl of pooled extract, 100 µl of increasing concentrations of corticosterone standard were added in the range used for the standard curve. The diluted pool was assayed alone to determine endogenous hormone levels. The percent recovery was calculated using the following formula: amount observed / amount expected x 100%; where amount observed is the value obtained in the spiked sample and amount expected is the calculated amount of corticosterone standard added plus the amount of endogenous hormone in the unspiked sample. The percent recovery is presented as mean ± SEM. The graphs were plotted as hormone added vs. hormone recovered and regression analyses were used to determine if there was a significant relationship between them.

Statistical Analysis

Statistical analyses were performed using R 2.15.1 (R Core Team, Vienna, Austria) and Excel (Microsoft Office Excel 2007, Microsoft, Redmond, WA, USA).

Baseline concentrations of CORT were determined on an individual basis in the same manner as the reproductive hormones, as described in chapter 1. Significant peaks were defined as values above baseline + 1.5 SD. The peak maximum value was calculated as the difference between the highest measurement of the peak and the baseline concentration measured in ng CORT per g dry fecal matter.

Within each cycle, the time between the maximum peaks of each individual hormone (CORT, E2, T and P) was measured in days. Differences in maximum hormone peak values, and timing between hormone peaks within a cycle were compared between the first and second cycles of all animals (n=28) and between productive and non-productive cycles of egg laying animals (n=4) using paired T-tests for repeated measures on the same animals. P values < 0.05 were considered significant.

Hormone metabolite values were graphed against time (October 1, 2011 – July 3, 2012), recognizing the fact that each measurement represented a sample pooled over four days and

that hormones appear in the feces some days later than their presence in circulating blood (Klasing, 2005; Millspaugh and Washburn, 2004).

RESULTS

CORT assay validation

Extraction of exogenous CORT resulted in fecal extraction efficiency of 96.6 ± 4.2 % (mean ± SE). Serial dilutions of pooled fecal extracts showed parallel displacement with the standard curve (r= 0.97, P< 0.01; Figure 2.1). The intra-assay CV at 50% binding was 5.6% and inter-assay CV's were 17.0% and 15.0% at 25% and 65% binding, respectively. The recovery of known concentrations of CORT was 85.4 ± 4.4%. The measured hormone concentrations in the spiked samples correlated with the expected concentrations (r= 0.99, P < 0.01) (Figure 2.2).

Figure 2.1 Recovery of exogenous corticosterone from pooled fecal extracts of female veiled chameleons

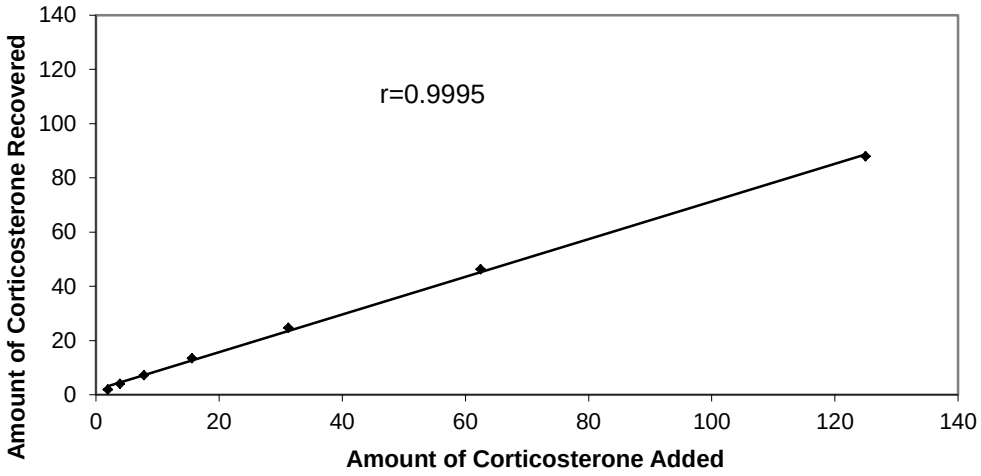
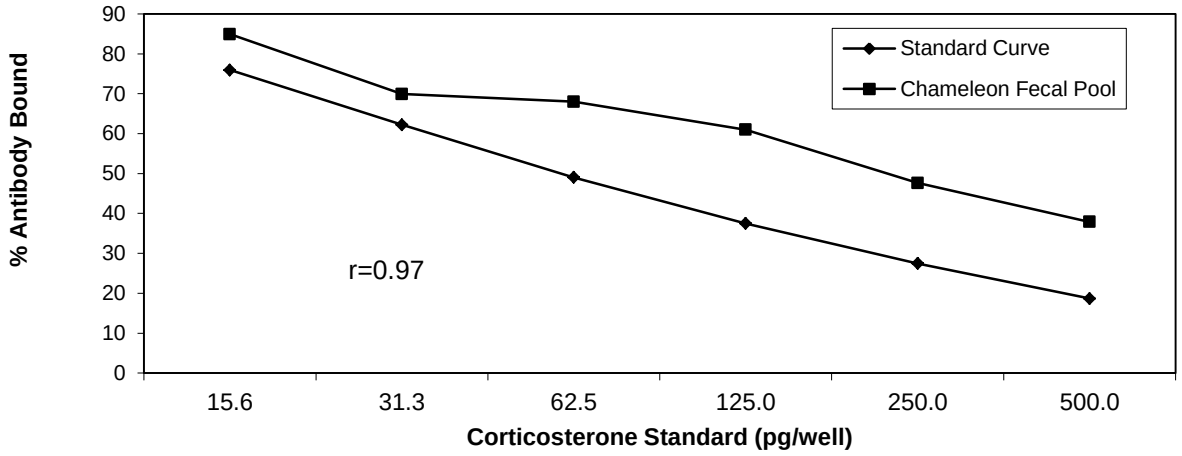


Figure 2.2 Parallelism for serial dilutions of pooled fecal extracts of female veiled chameleons against the standard curve.



Adrenal and reproductive activity throughout the reproductive cycle

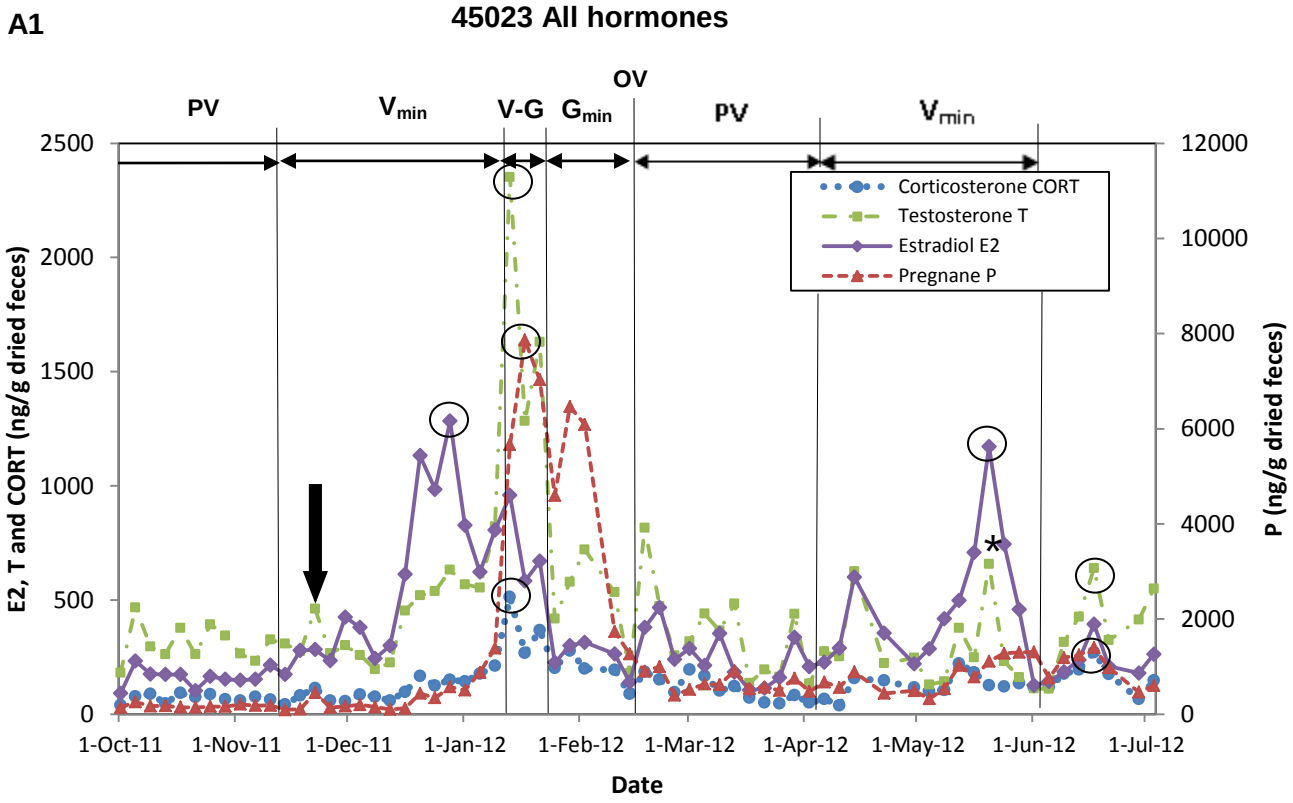
Hormone profiles indicated a cyclical pattern of CORT, consistent with patterns observed in the reproductive hormones (Figure 2.3). Levels of CORT began to increase during early-mid vitellogenesis, peaked in late vitellogenesis (when follicles reached mature size), or within the vitellogenic/gravid overlap, and remained elevated until just prior to oviposition (in productive cycles). In animals that did not ovulate, CORT levels returned to baseline following the peak. In some of the female's cycles, significant peaks in CORT, and T occurred during the early vitellogenic phase, but this trend was not consistent between subsequent cycles, and thus was not considered to be associated with reproductive events.

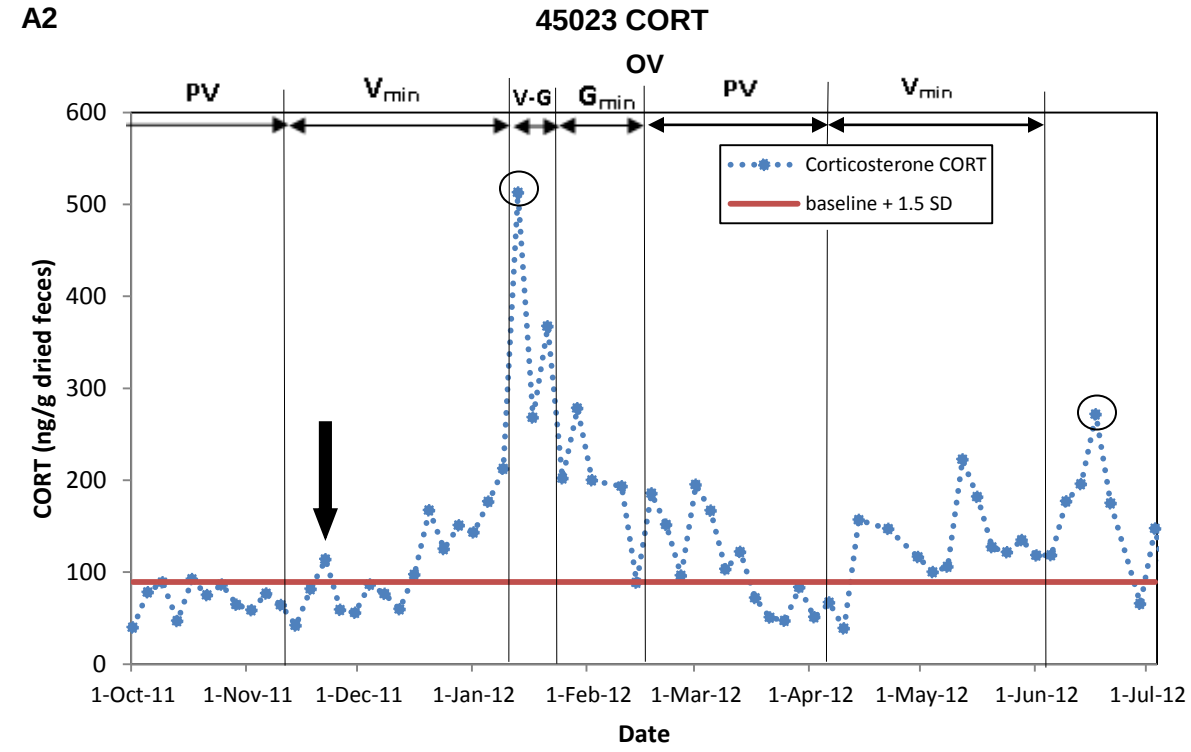
Hormone patterns consisted of complexes of hormone peaks, in which a peak in E2 was followed by distinct peaks in CORT, P and T; which occurred in close proximity to each other. The majority of the females underwent two cycle complexes within the study period. The mean number of days between hormone maximum peak values for all animals (n=28) for cycles 1 and 2 is presented in Table 2.1, and between productive and non-productive cycles for egg-laying

animals (n=4) in Table 2.2. No significant differences were found in the number of days between hormone peak maximum values for each hormone combination (CORT-P, CORT-T, CORT-E2, E2-P, etc.) between cycles 1 and 2, or between non-productive and productive cycles. Therefore, the mean number of days between each of the hormone peaks within a cycle complex can be approximated as follows: E2 and P: 26 days, E2 and T: 31 days, and E2 and CORT: 32 days, CORT and P 1.7 days, CORT and T: 2 days, and T and P: 1.5 days.

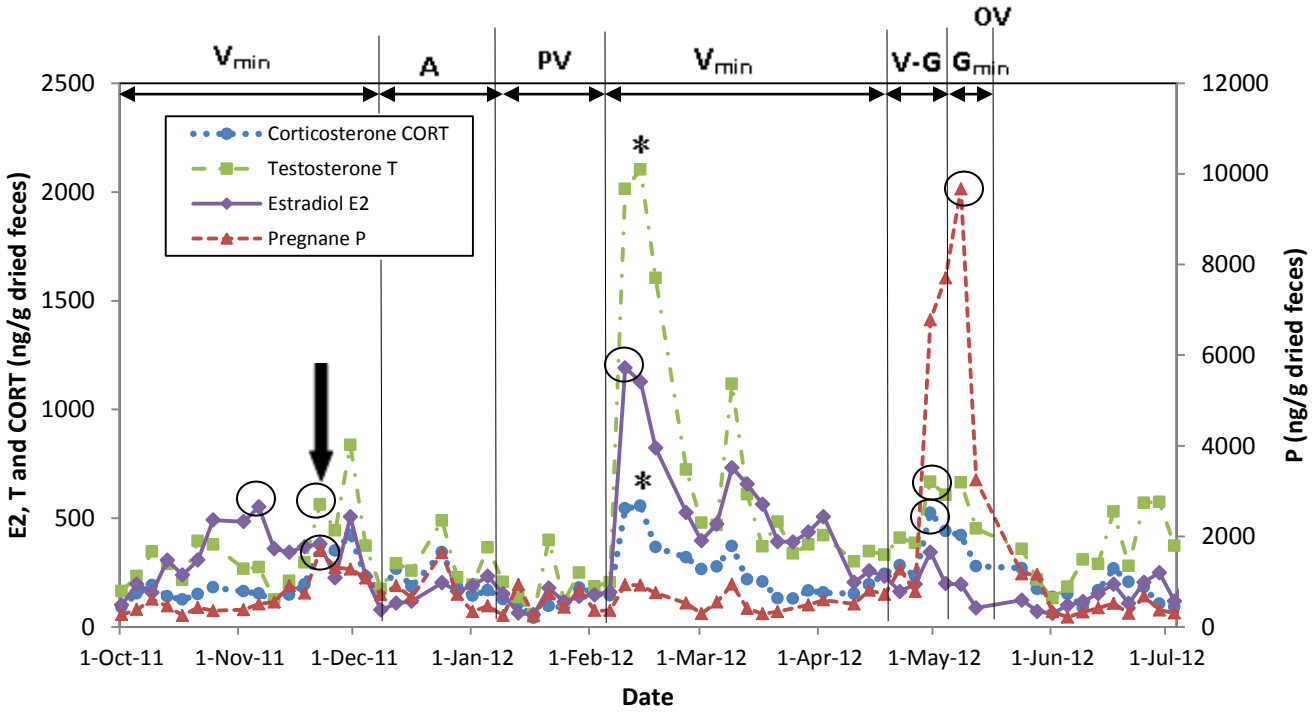
The maximum peak hormone values for productive and non-productive cycles for individual egg-laying animals are presented in Table 2.3, and the mean values are represented graphically in Figure 2.4. There was a significant difference in CORT maximum peak values between ovulatory and non-ovulatory cycles ($p=0.04$), but not for any of the other hormones based on the four animals that were evaluated. Although there is a clear difference between peak P levels between when assessed graphically (Figure 2.4). Peak values of P were approximately 20-fold (or above) higher than baseline values in productive cycles, compared to 8.5-fold (or below) for non-productive cycles.

Figure 2.3 Fecal hormone metabolite patterns of two female veiled chameleons with ovulatory and non-ovulatory cycles

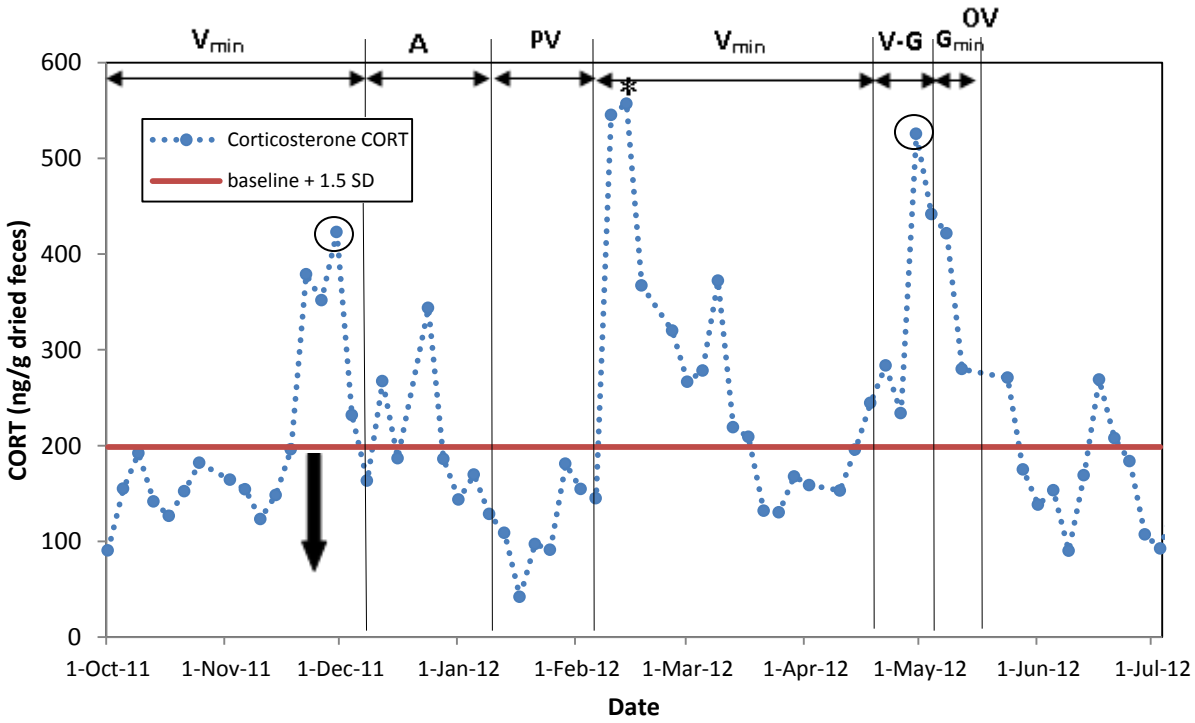




B1



B2



Relationships between fecal metabolite patterns of estradiol (E2), progesterone (P),

testosterone (T) and corticosterone (CORT) (A1, A2), and CORT patterns relative to baseline values +1.5 standard deviations (SD) (B1, B2), plotted against time (October 1, 2011 – July 3, 2012) for two animals (A; 45023, and B; 45016) that had both productive and non-productive cycles. Stages of the reproductive cycle are identified with arrows at the top of the graph: PV = previtellogenic, Vmin = minimal duration of vitellogenesis, V-G = vitellogenesis/gravid overlap including the event of ovulation, Gmin = minimal duration of gravid stage. Solid, diamond (♦) = E2; dash-dotted, square (■) = T; dashed, triangle (▲) = P; round dotted, circle (●) = CORT. Maximum peak hormone values associated with biological events are indicated with a circle; asterix mark maximum hormone values in a given cycle that were not associated with biological events. Large arrow indicates the date of the move (November 23, 2012).

Table 2.1 Days between maximum peak hormone values for all animals

	CORT-P	E2-CORT	CORT-T	E2-T	E2-P	T-P
C1	2.77	-32.43	0.15	-32.29	-30.31	2.4
C2	0.57	-22.29	1.29	-21	-21.71	-0.71
Mean (C1, C2)	1.67	-27.36	0.72	-26.65	-26.01	0.84
Mean of the Differences	3.38	-10.14	-1.04	-10.52	-7.08	3.52
p-value	0.14	0.12	0.64	0.13	0.28	0.34

Mean number of days between maximum peak values for fecal metabolites of CORT, P, E2 and T during the first and second cycle complexes (C1 & C2, respectively) of twenty-eight female veiled chameleons. Negative numbers indicate that the maximum peak of the first hormone preceded the second. $p<0.05$ was considered significant.

Table 2.2 Days between maximum peak hormone values in egg-laying animals

	CORT-P	E2-CORT	CORT-T	E2-T	E2-P	T-P
p	-2	-44	1	-17	-46	-3
np	3	-20	3	-43	-17	0
Mean (p, np)	0.5	-32	2	-31.5	-30	-1.5
Difference (p-np)	5	24	2	26	29	3
p-value	0.14	0.23	0.60	0.22	0.21	0.59

Mean number of days between maximum peak values for fecal metabolites of CORT, P, E2 and T during the productive (p) and non-productive (np) cycle complexes of four female veiled chameleons. $p<0.05$ is significant.

Table 2.3 Hormone peak maximum and baseline values for productive and non-productive cycles of egg-laying animals

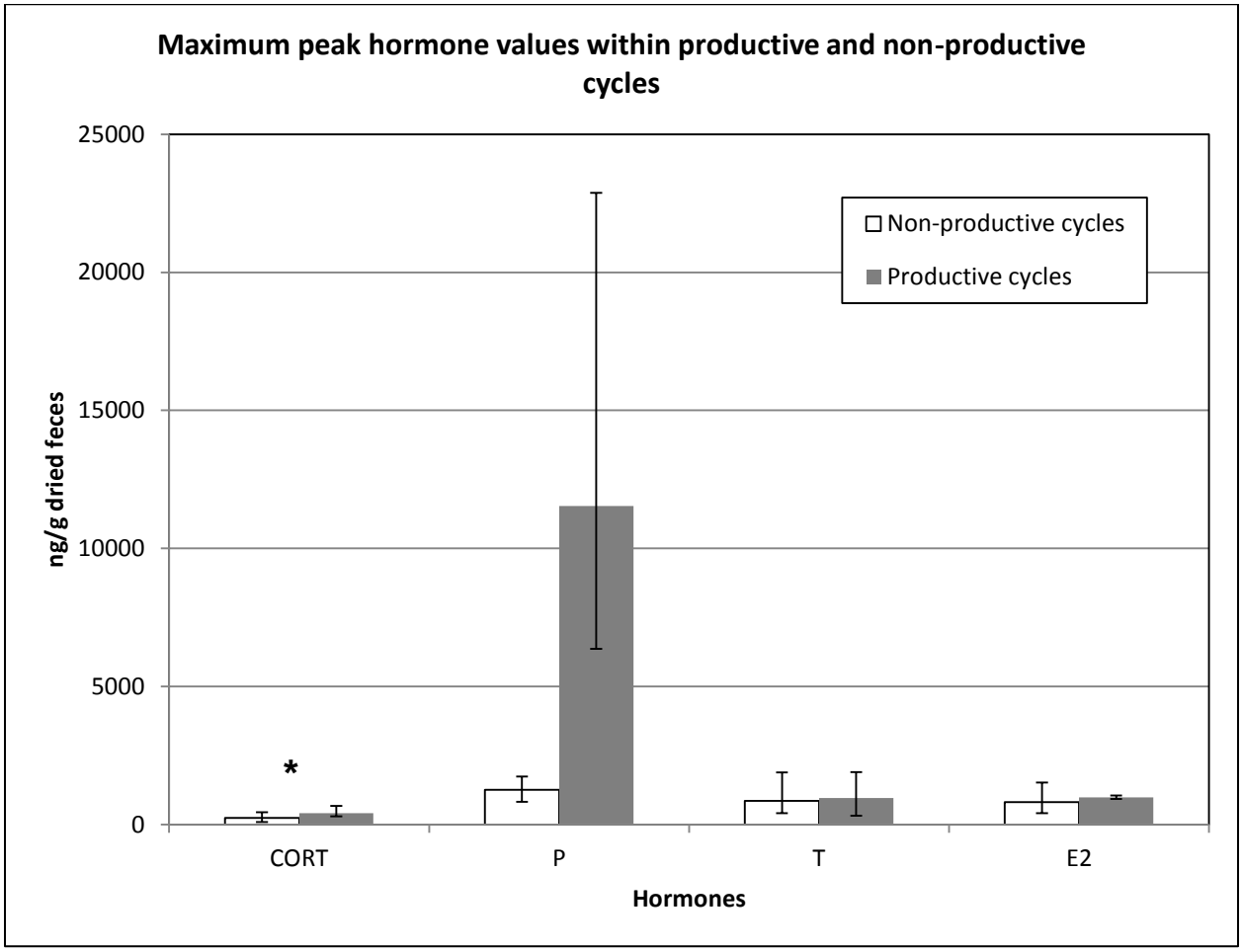
A	Peak maximum (baseline)			
Animal	E2	CORT	P	T
A	925.03 (179.30)	381.84 (65.4)	7569.78 (146.5)	1892.26 (229.8)
B	948.74 (73.1)	672.92 (91.8)	22882.66 (309.4)	1233.77 (183.1)
C	997.37(111.87)	298.66 (95.29)	6359.97 (325.9)	321.34 (199.49)
D	1050.37 (141.1)	296.2 (145.4)	9347.395 (317.8)	399.47 (268.2)
E	406.30 (104.6)	232.1 (76.36)	4353.64 (227.7)	277.35 (204.8)
Average	865.56 (121.99)	376.34 (94.85)	10102.69 (265.46)	824.84 (217.08)
SD	261.2 (40.14)	174.11 (30.72)	7371.79 (77.3)	714.28 (33.13)

B	Peak maximum (baseline)			
Animal	E2	CORT	P	T
A	813.77 (179.3)	140.6 (65.4)	1105.3 (146.5)	409.68 (229.8)
B	1517.63 (73.1)	444.31 (91.8)	1735.5 (309.4)	1890.44 (183.1)
C	515.46 (111.87)	94.12 (95.29)	816.65 (325.9)	545.57 (199.49)
D	411.55 (141.1)	277.49 (145.4)	1367.8 (317.8)	568.63 (268.2)
Average	814.61 (126.34)	239.13 (99.47)	1256.31 (274.9)	853.58 (220.15)
SD	498.73 (44.97)	157.38 (33.4)	390.79 (85.86)	694.79 (37.42)

Peak maximum values and calculated baseline values (in brackets) for fecal CORT, P, E2 and T

metabolites during productive (A) cycles in five female veiled chameleons (*Chamaeleo calyptratus*) and non-productive (B) in four female veiled chameleons. All values are in ng/g dried feces.

Figure 2.4 Mean maximum peak hormone values during productive and non-productive cycles of female veiled chameleons



Calculated mean maximum peak values for fecal metabolites of CORT, P, E2 and T during productive and non-productive cycles based on four female veiled chameleons. Error bars indicate minimum and maximum values of each hormone. Asterisks mark significant differences ($p < 0.05$) between hormone concentrations according to paired t-test.

Response to change in environments

It was not possible to attribute peak values of CORT levels that occurred following the change in external environments solely to activation of the HPA axis as the result of a stress response, as it turned out that cycle stage was a confounding variable. This was illustrated by the fact that significant peaks in CORT levels occurred in 9/28 animals; of which 8/9 were in mid-late phases of vitellogenesis, and 1/9 in early vitellogenesis. Additionally, significant peaks in P and/or T levels were noted to occur at the same time.

DISCUSSION

The performance of the specific assay chosen to measure corticosterone in this study was validated with parallelism, accuracy and precision tests. These tests showed that the assay reliably measured the hormones present in the fecal extracts without being influenced by components within the extract.

Adrenocortical activity, and subsequently, glucocorticoid levels can vary in a particular animal with age, reproductive status, sex, diet, and season (Millspaugh and Washburn, 2004; Moore and Jessop, 2003; Woodley, 2002). In the current study, all of the animals were born from the same clutch, therefore eliminating any effect of age and/or genetic factors. In addition, all of the animals were female and maintained under identical conditions, thereby eliminating any sex, diet and seasonal effects. Therefore, an assessment of variation in glucocorticoid levels could be made between the various stages of the reproductive cycle, and more specifically, between ovulatory and non-ovulatory cycles without interference from those confounding factors.

In the current study, we describe a cyclical pattern in CORT levels which varied according to reproductive stage in female veiled chameleons. This is consistent with other studies reporting a cyclical pattern of corticosterone throughout the reproductive cycle in other reptile species (Dauphin-Villemant et al., 1990; Grassman and Crews, 1990; Wilson and Wingfield, 1992). Based on our data, we identified the following pattern: CORT levels rose during mid-late vitellogenis, peaked during the vitellogenic/gravid overlap phase, when follicles had reached mature size, and remained elevated until just prior to oviposition. Similar patterns

were observed throughout the reproductive cycles of female *U. stansburiana* (Wilson and Wingfield, 1992); whereby corticosterone production increased throughout the vitellogenic phase, and remained elevated in individuals that contained oviductal eggs. Additionally, Dauphin-Villemant et al. (1990) observed low circulating levels of corticosterone during the vitellogenic phase of female *L. vivipara*; which gradually increased during the first half of gestation, peaked significantly in late gestation, then fell abruptly prior to parturition. In contrast, Grassman and Crews (1990) found that plasma levels of corticosterone were highest during the previtellogenic period, decreased during vitellogenesis; when estradiol levels started to increase; and remained low during the gravid period of *C. uniparens*.

Also, in some species, plasma levels of corticosterone are elevated during the breeding season compared to the non-breeding season, but variations over time (month) or between different stages of the reproductive cycle do not occur. This has been reported in common geckos; *Hoplodactylus maculates* (Girling and Cree, 1995), and American alligators; *Alligator mississippiensis* (Guillette et al., 1997). Moreover, there are species that do not display elevated levels during the breeding season, such as *P. barbata* (Amey and Whittier, 2000).

Altogether, the above studies indicate that the relationship between glucocorticoid production and reproductive activity is species-specific, and thus cannot be universally defined in reptiles. Additionally, the source of glucocorticoid production and secretion during periods of reproductive activity remains unclear, so it is not possible to determine whether glucocorticoid levels are elevated as a result of the stress associated with reproduction, or if it is necessary to facilitate biological events.

In the current study, the limited number of animals that underwent productive cycles precluded our ability to define differences in maximum peak levels of CORT during productive and non-productive cycles with statistical confidence. However, the limited data available suggests that maximum peak levels of CORT are elevated in cycles where animals ovulate compared to non-productive cycles. A similar trend was observed in maximum peak progesterone levels in both the current and previous study of the same species by Kummrow et al. (2010c,d). Kummrow et al. (2010c) proposed that ovulation occurred with the decreasing estrogen: progesterone ratio and that progesterone peaks during mid-gravidity and falls prior to oviposition. It is logical to assume that this dramatic increase in progesterone following ovulation is the result of corpora lutea formation on the ovaries, and that progesterone produced therein is

responsible for the maintenance of the gravid state (Callard and Ho, 1980; Callard et al., 1992; Shanbag et al., 2001). The results of the current study indicate a close relationship between the timing of peaks in P, T and CORT (within 3 days of each other). Therefore, it is possible that there is an interaction occurring between these three hormones around the time of ovulation that facilitates this event to occur. Since the maximum peaks in CORT occurred in the timing between ultrasound exams, during which the transition from follicle to egg occurred (the vitellogenic-gravid overlap phase), it remains unclear whether this surge facilitated or occurred as a result of the ovulatory process. In order to assess this more accurately, visualization of the reproductive tract could be done on a more frequent basis to narrow down the date of ovulation, and a comparison of corresponding levels of the individual hormones in the days immediately prior to and following ovulation would reveal if they are occurring prior to or following ovulation.

The elevated levels of corticosterone following ovulation may facilitate the natural retention of eggs during the gravid phase in order to prevent early expulsion. Dauphin-Villemant et al. (1990) found that parturition was significantly advanced in adrenolectomized female *L. vivipara*, and significantly delayed in animals treated with exogenous corticosterone in late gestation. Along the same lines, Jones et al. (1983) found that argonine vasotocin (AVT)-induced uterine contractions were inhibited in *A. carolinensis* with high circulating levels of epinephrine (a downstream product of elevated levels of corticosterone in response to stress), whether of endogenous or exogenous origin. Additionally, Shanbhag, et al. (2001) speculate that prolonged egg retention observed in *C. versicolor* could have been accomplished by the secretion of progesterone and corticosterone by the adrenals under “stressful” conditions; which in turn inhibit the oviductal contractions associated with oviposition.

In the present study, it was not possible to determine whether the females underwent a stress response in response to the change in environments. This was due to the fact that 1) the stage of the reproductive cycle that the animals were in was a confounding factor, and 2) there was no control group to compare population baseline averages to. However, there is evidence to suggest that the animals did not undergo a stress-response. For instance, the presence of large follicles on the ovaries of the majority of females at the initiation of ultrasound examinations in February indicates that vitellogenesis was not inhibited as a result of increased glucocorticoid production. A couple of studies have demonstrated that elevated levels of glucocorticoids have an anti-gonadal on effect. In *Mabuya carinata*, FSH-stimulation of the ovaries; including FSH-induced germinal bed activity, vitellogenesis, and steroidogenic activity

of the ovary; was inhibited by the administration of exogenous corticosterone (Nijagal and Yajurvedi, 1999). A failure to produce eggs in response to low-humidity conditions (both in the wild and captivity) in *A. carolinensis* is speculated to occur as the result of activation of the HPA axis in response to dehydration, and this results in the down-regulation of the HPG axis (Summers, 1988). Therefore if corticosterone levels were significantly elevated as the result of a stress-response we would expect to see inhibition of vitellogenesis. Additionally, our analysis of hormone patterns reveal that an increase in corticosterone occurs in the mid-late stages of vitellogenesis in female veiled chameleons, so elevations observed in some of the females may have been artifacts of reproductive activity since they were accompanied by peaks of progesterone and testosterone metabolites.

It is also unlikely that failure to ovulate was the result of the change in environments since the move occurred early on in the reproductive cycles of the majority of females, and hormone patterns indicate that elevated levels of corticosterone naturally occur around the time of ovulation.

Altogether, it is unlikely that the high incidence of follicular stasis in the study population was the result of a stress-response elicited by the move. However, in order to rule out this possibility, a control group that did not experience a change in environment would be needed to compare of baseline levels of CORT to, but this was not possible in the current study.

CONCLUSION

With this study we were able to characterize the cyclical patterns of corticosterone that occur throughout the reproductive cycle of female veiled chameleons. Further investigation into the mechanisms that trigger and inhibit ovulation in this species is needed as are methods of inducing ovulation in animals that failed to do so. Additionally, studies are needed to determine the impact of (changes to) the environment has on glucocorticoid production and if this is related to a high incidence of egg-binding in veiled chameleons.

CHAPTER THREE

Investigating methods of overcoming follicular stasis in female veiled chameleons (*Chamaeleo calyptratus*)

INTRODUCTION

Reproductive disorders are a common cause of death in captive female reptiles (Cuadrado et al., 2002; Rivera, 2008; Sykes, 2010). The most commonly observed condition, known as egg binding refers to the retention of eggs within the reproductive tract beyond the length of a normal cycle (Cuadrado, 2002; Kummrow et al., 2010c,d; Rivera, 2008; Sykes, 2010). It can be further classified based on the location of retention within the reproductive tract; on the ovary, failure of follicles to ovulate and recede normally is known as *follicular stasis*, and within the oviducts, lack of oviposition of fully formed eggs is known as *dystocia*. Retained eggs can cause inflammation, fluid build-up, compaction of the digestive tract, egg yolk coelomitis following rupture of a follicle or egg, and, if left untreated, can result in death of the animal (Castle,1990; Rivera, 2008; Schumacher and Toal, 2001; Stacy et al., 2008).

Although dystocia can be readily treated (and often resolved) with the administration of arginine vasotocin (AVT) or oxytocin to induce uterine contractions, and subsequently oviposition, treatment of follicular stasis has been limited to individual follicle aspiration; a perilous procedure which carries a high risk of follicle rupture, and fails to prevent future occurrences (Rivera, 2008), or removal of part or all of the reproductive tract (Backues and Ramsay, 1994; Kramer, 2006; Sykes, 2010). Ovarie- or hysterectomy are good options when the animal is not required for breeding, but far less desirable for animals meant to be involved in breeding programs. Little research has focused on finding alternative prevention and/or treatment options for follicular stasis, though this may be possible by means of hormonal manipulation and feed restriction.

Inhibition of gonadal activity and follicular development by administration of exogenous hormones has been examined in a few reptile species. The effects of progesterone (administered over several days) on follicular development has been investigated in *Scleroporus cyanogenys* (Callard et al., 1972a,b; Callard et al., 1973), *Chamaeleo pumilus* (Veith, 1974), *Xantusia vigilis* and *Lacerta sicula* (Yaron and Zidler, 1978). Results of the above studies indicate that progesterone has anti-gonadal effects; arresting follicular development, inducing yolk regression, causing decreases in ovarian and oviductal weights, and preventing ovulation (although it is dependent on what stage of development the follicles are in at time of administration). Callard et al. (1972b) also examined the effects of prolactin in *S. cyanogenys* and found that it had inhibitory effects on ovulation and oviductal growth, but these effects were

less pronounced compared to animals that received progesterone. Tamoxifen (an estrogen blocker) implanted intracoelemically prevented follicular development and oviposition for one entire breeding season in leopard geckos; *Eublepharis macularius* (DeNardo and Helmenski, 2001), but conversely, had no effect in female veiled chameleons; *Chamaeleo calyptratus* (Kummrow et al., 2010a). In the same study by Denardo and Helmenski (2001), indomethacin (a prostaglandin E antagonist) prevented follicular development, but caused severe side effects in leopard geckos. Jones et al. (1990) observed similar antigonadal effects of indomethacin in green anoles; *Anolis carolinensis* but no side effects resulted from its use. Anti-gonadal effects of GnRH have been observed in humans, mice (Alkis et al., 2011), and cockatiels (Millam and Finney, 1994); however, no studies have investigated the effects of GnRH analogues to inhibit follicular development in reptiles.

Ovulation induction of ripe follicles has been successfully accomplished using mammalian and reptilian (turtle) variations of follicle stimulating hormone (FSH) and luteinizing hormone (LH), alone or in combination in a variety of lizard species, including: *A. carolinensis* (Jones et al., 1988; Jones et al., 1990; Licht 1970; Licht and Crews, 1975; Licht and Tsui, 1975), garden lizards; *Calotes versicolor* (Shanbag and Prasad, 1993), common side-blotched lizards; *Uta stansburiana* (Licht, 1970), Texas horned lizards; *Phrynosoma cornutum* (Burns and Richards, 1974), and western fence lizards; *Sceloporus occidentalis (*Licht, 1970). All studies on lizards have shown that the potency of LH is minimal compared to FSH in stimulating ovarian and oviduct growth, and there is only one report of ovulation induction from the administration of turtle LH in *A. carolinensis* (Licht and Crews, 1975), whereas FSH has a high success rate of inducing ovulation. A few studies have also examined the effects of gonadotropins on the ovaries of turtles and snakes (Callard et al., 1976; Chan and Callard, 1974; Lance and Callard, 1978; Licht, 1972). Conflicting results have been observed in turtles, and the snake, *Natrix fasciata,* where LH appears to be more than or equally as potent as FSH*.* Studies on amphibians, including the Indian skipper-frog; *Rana cyanophlyctis* and northern leopard frog; *Rana pipiens* have shown that progesterone is capable of inducing ovulation on sections of ovary *in vitro* (Edgren and Carter, 1963; Ramaswami and Lakshman, 1958; Wright, 1961 & 1971). Additionally, prostaglandin (PG) F has been shown to increase ovulation and spawning rates in a population of teleost fish, *Piaractus mesopotamicus* with low spawning rates. However, the use of progesterone or PGs for ovulation induction has not been studied in lizards, nor has ovulation induction in animals with follicular stasis been attempted.

Female reptiles in captivity produce larger clutches with larger eggs compared to wildlife counterparts, in large part due to the constant provision of food (Cuadrado, 2000; DeNardo et al., 2002; Rivera, 2008). Egg production is an energy-demanding process, and clutch and egg size is related to the body condition of the female (DeMarco, 1989; Rhen et al., 2006). Rhen et al. (2006) state that the energy required for this process can come from stored resources (capital), those acquired during reproduction (income), or a combination of both; and is proportional to the energy available. So, in theory, if a female is in a negative energy state, and is unable to acquire additional income energy, she will down-regulate egg production in order to conserve resources. This phenomenon has been observed in laying hens; molting induced by feed restriction results in the hens shedding their feathers, and follicular regression occurring on the ovary (Koch, 2005). As a result, egg-laying is put off until the molt is complete. In addition, feed restriction may elicit a stress response, resulting in activation of the hypothalamic-pituitary-adrenal (HPA) axis, and downstream, increased production of progesterone and corticosterone from the adrenal cortex. As previously mentioned, elevated progesterone over a long period of time has anti-gonadal effects, and could also contribute to follicular atresia. To the author's knowledge, no studies have been conducted in which female reptiles have been fed a restricted diet as a means of altering egg production.

The objectives of this study were to: 1) attempt to resolve follicular stasis, by inducing ovulation and/or atresia/reabsorption of yolk in affected animals by means of hormonal manipulation and/or feed restriction, and 2) examine the usefulness of readily available human-based contraceptives at preventing follicular development.

MATERIALS AND METHODS

Animals

The animals used in this portion of the study were subgroups of the colony discussed in chapter 1. Husbandry conditions and detailed information on the methods used to evaluate fecal reproductive hormone metabolites, ultrasound images, and male introduction reactions are described in detail in chapter 1; and fecal adrenal hormone metabolites in chapter 2.

Ovulation induction

Animals were diagnosed with follicular stasis if: 1) they had large round structures in the reproductive tract that did not change significantly in size between three consecutive ultrasound exams (conducted over a 3 week period), and 2) they did not display non-receptive behaviour towards conspecifics (described in detail in chapter 1). Based on these criteria, 8 animals were excluded from the trial because they were suspected of having eggs (6/8), had recently laid a clutch (1/8), or had been spayed (1/8). The remaining animals (N=21) were divided into three treatment groups, each consisting of animals with 1) increases and 2) decreases in the size of follicles, in combination with those that a) did or b) did not react to males. The 3 treatment groups were as follows: group 1 (n=7) received two intramuscular (i.m.) injections of chicken GnRH II (cGnRH II) at a dose of 100 µg/kg dissolved in 20 µl saline (American Peptide Company Inc., cat# 54-8-24), 12 hours apart; group 2 (n=7) received progesterone at a dose of 1mg/kg (Sigma cat# P8783-1G) dissolved in 40 µl saline, followed by prostaglandin F2α at a dose of 10 µg/kg (Sigma cat#P5069-1MG) dissolved in 20 µl saline, i.m., 12 hrs later; and group 3 (n=7) served as a control and did not receive any treatment.

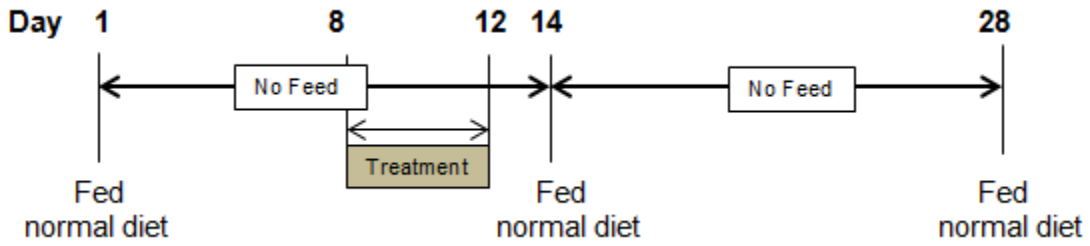
Ultrasound exams and fecal reproductive hormone metabolite analysis were used to verify the success of the treatments (methods described in chapter 1).

Feed restriction

Twenty-one females participated in a feed restriction trial, combined with (or without) hormonal manipulation conducted over a 4 week (28 day) period from July-August 2012 (Figure 3.1). Animals were fed normal diets on days 1, 14, and 28, but did not receive any food between those dates; whereas the animals not participating in the trial (n=3) remained on the normal diet and feeding schedule (3X/week) throughout the four week period. The females on the restricted diet (n=21) were divided into three groups, receiving treatment for 5 consecutive days commencing on day 8 of the trial. This point in time was selected to allow the animals' metabolisms to adjust to the reduction in feed, in order to eliminate issues with absorption of the drug. Oral administration was selected to ensure that the drug would be absorbed quickly, and it did not require repeated injections with needles (although there is no evidence to suggest that one method would cause more discomfort/stress over another).

The first group (n=7) received an oral dose of 1 mg progesterone (Sigma cat# P8783-1G) dissolved in 1 ml saline, the second (n=7), 1 ml saline with no hormone additive, and the third group (n=7) served as a control and did not receive any handling/treatment. Animals were weighed weekly during the course of the trial, and ultrasound examinations were conducted every 2 weeks.

Figure 3.1 Feed restriction trial experimental design



Females that took part in the feed restriction trial (n=21) received normal diets on days 1, 14 and 28, with no food in between those dates. Treatments (progesterone, saline, or no treatment) were administered from days 8-12.

Contraception

Four animals received hormone contraceptive treatment during the time the females resided at the Toronto Zoo. The prerequisite for participation in the contraceptive study required the animals to oviposit eggs prior to receiving treatment, in order to ensure that the animals were in previtellogenesis, when basal levels of circulating sex steroids can be found. Although five animals laid eggs throughout the study period, one was undetected at the time as no digging behaviour was observed and eggs were not discovered until a couple of months later. The animals received two i.m. shots of 1) 1 mg/kg Lupron Depot (leuprolide acetate; a GnRH agonist) (Abbott Endocrine; Abbott park, Illinois, United States) in 30 µl saline (n=1) or 2) 10 mg/kg Depo Provera (medroxyprogesterone acetate; a progesterone agonist) (Pfizer; New York, New York, United States) in 20 µl saline; a progesterone agonist (n=3), at two and four weeks following oviposition. These hormones were selected based on their anti-gonadal properties when administered over long periods of time in humans and other mammalian species, and doses based on wildlife recommendations (2-5 mg/kg for Depo Provera, and 0.1-1 mg/kg for

Lupron Depot).

Statistical Analysis

Statistical analyses were performed using SAS 9.1.2 (SAS Institute Inc., Cary, NC, USA) and Excel (Microsoft Office Excel 2007, Microsoft, Redmond, WA, USA).

The change in follicle SA from the start of the trial to the end of the trial was calculated by subtracting the initial SA from the final SA values. Increases and decreases in SA were compared to changes in bodyweight, and stage of reproductive cycle to assess whether a relationship existed among these factors.

The effect of initial weight on final weight (weight loss) was determined using a standard analysis of covariance, with treatment as a factor, initial weight as a covariate. This model adjusts the means for the treatments to a common covariate value (mean of data set) prior to comparing relative weight loss in each of the treatment groups.

The change in weight (in g) was calculated by subtracting the mean initial weight (all animals) from the final weight (adjusted to the mean initial weight). The relative change in weight was calculated as the weight change (in g) divided by the mean initial weight X 100%.

The effect of treatment on relative weight change (adjusted for initial weight) was determined using a standard one-way ANOVA. A general linear mixed model was fitted to the data, using Proc MIXED (SAS version 9.1.2).

To assess ANOVA assumptions, residual analyses were performed, including formally testing the residuals for normality, using the four tests offered by SAS (Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises, and Anderson-Darling). The residuals were plotted against the predicted values. Such analyses may reveal the need for transformations, outliers or other factors to consider.

Biological data

Dates of oviposition were recorded, and necropsies were performed at the end of the study, at which time the reproductive tract was removed and evaluated. Follicles and/or eggs found therein were measured for length and width using electronic calipers (carbon-fiber composite digital caliper 6 inches; Traceable Calibration Control Company, Friendswood, Texas, United Sates; Fisher Scientific cat#15-077-958), and notes on colour and vascularity were recorded.

RESULTS

A high prevalence of follicular stasis was diagnosed in the study population, which prompted research into finding a method to resolve the condition and examine methods for the prevention that would not require surgical intervention.

Ovulation induction

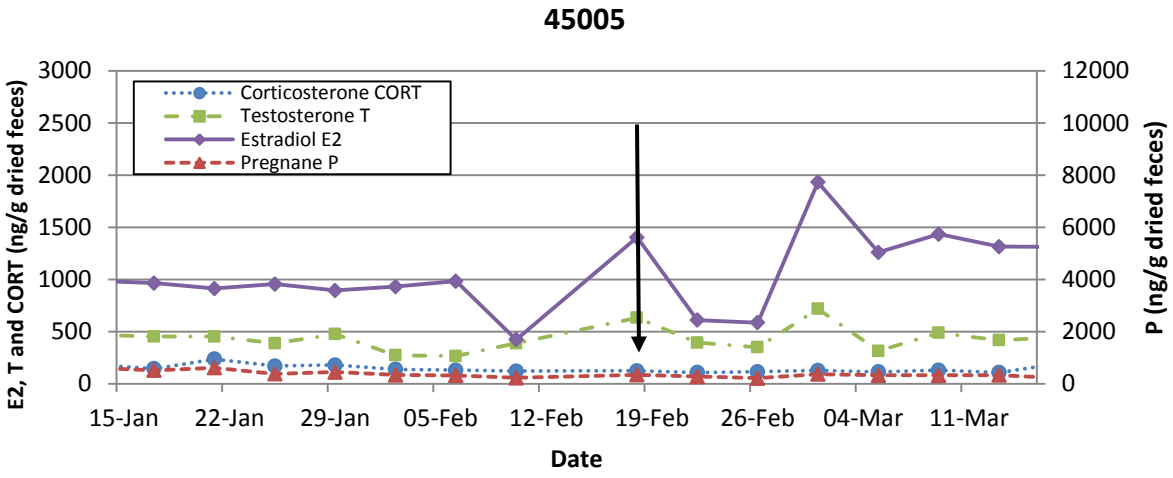
A pilot trial was conducted on one female diagnosed with follicular stasis (based on large/bloated belly for a prolonged period of time, no expected colour changes, and the large, round appearance of follicles, and fluid accumulation in the abdomen observed on ultrasound) early on in the establishment of the colony. The chameleon was given an injection of 100 µg folltropin-V (FSH equivalent to 400 mg NIH) in 0.2 ml saline subcutaneously in an attempt to induce ovulation. Due to the abnormal accumulation of fluid in the abdomen, and concern for the animals' health, the decision was made to perform an ovariectomy 3 days later. The FSH treatment was deemed unsuccessful and follicular stasis was confirmed upon identification and removal of the ovaries during surgery; multiple large follicles, along with a number of small follicles that had begun to develop were present on both ovaries. Although 1 animal was not enough to rule out FSH as a possible treatment option, it was unfeasible to use on the high number of animals involved in the induction trial, so c GnRH II was selected as an alternative, since it would have the downstream effect of increasing FSH release from the pituitary.

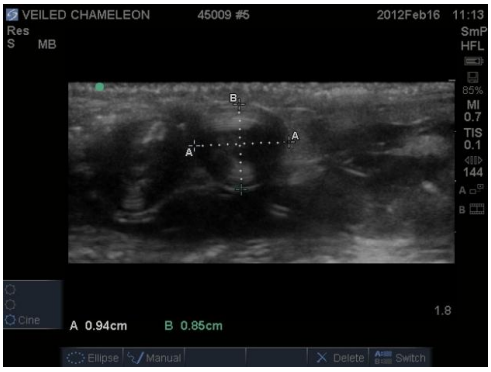
Ultrasonography, fecal reproductive hormone analysis and biological data revealed that ovulation (and subsequent oviposition of eggs) had not occurred as a result of hormonal

manipulation (Figure 3.2). Ultrasound exams following treatment indicated that morphological changes (i.e. transition from round to oval) had not occurred based on measurements and visual assessments of structures within the reproductive tract. The average length of the gravid period in veiled chameleons is 2-4 weeks (Kummrow et al., 2010c), and no animals were observed digging or laying eggs within several weeks (>8 weeks) following treatment. Analysis of fecal reproductive hormone metabolite profiles confirmed that ovulation had not occurred, as there was no surge in P levels (>20-fold above baseline values) following treatment. Additionally, there were no significant increases in the other hormones measured (E2, T and CORT), which suggested that the treatments did not affect endogenous hormone production.

Figure 3.2 Response of female veiled chameleons to ovulation induction treatments

A1





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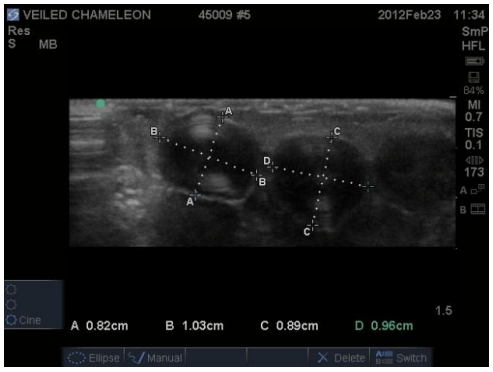

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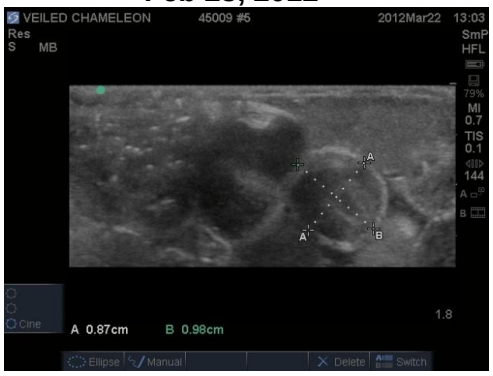

Mar 22, 2012

Measurement	16-Feb-12	23-Feb-12	8-Mar-12	22-Mar-12
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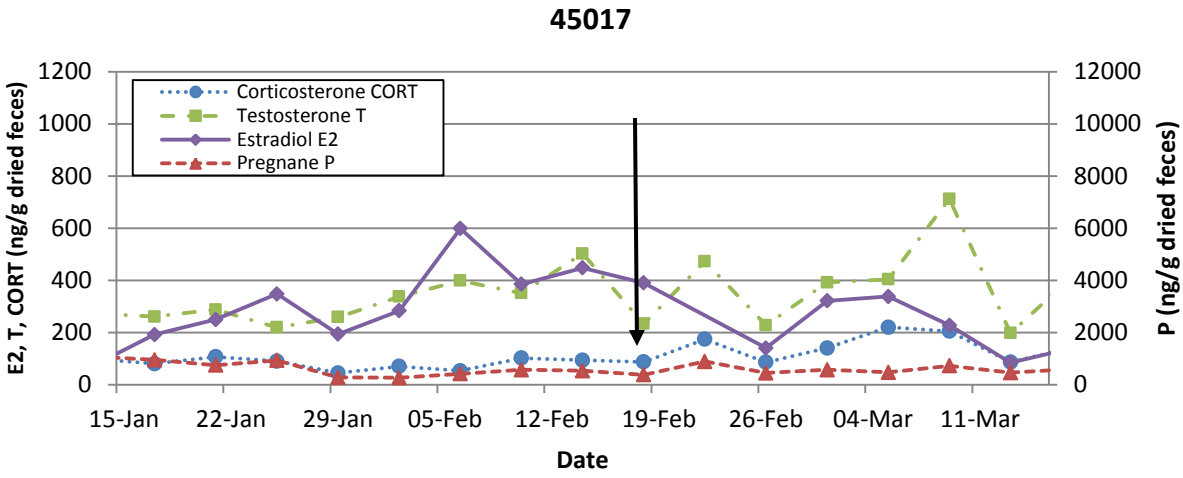
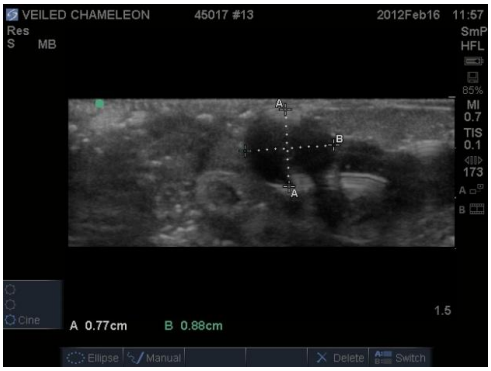
Width	0.85	0.8	0.62	0.76
L:W	1.11	1.33	1.29	1.36

A2





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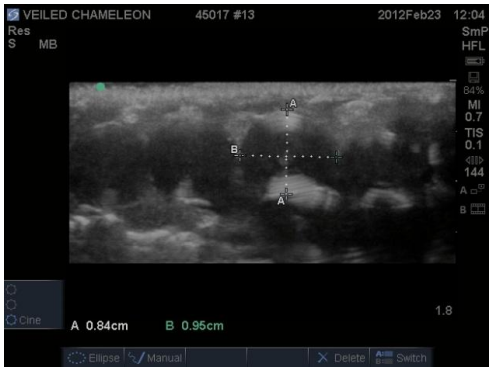

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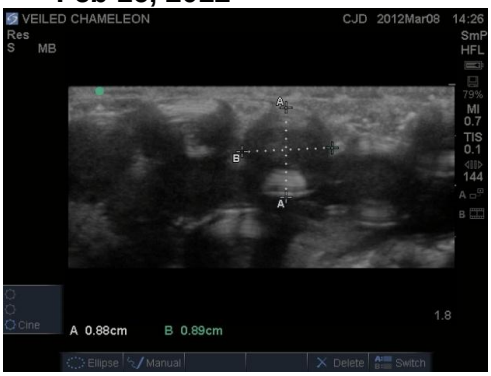

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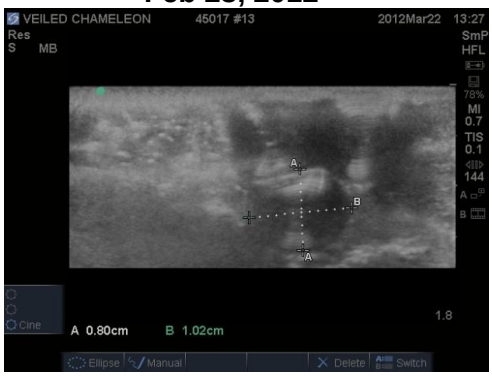
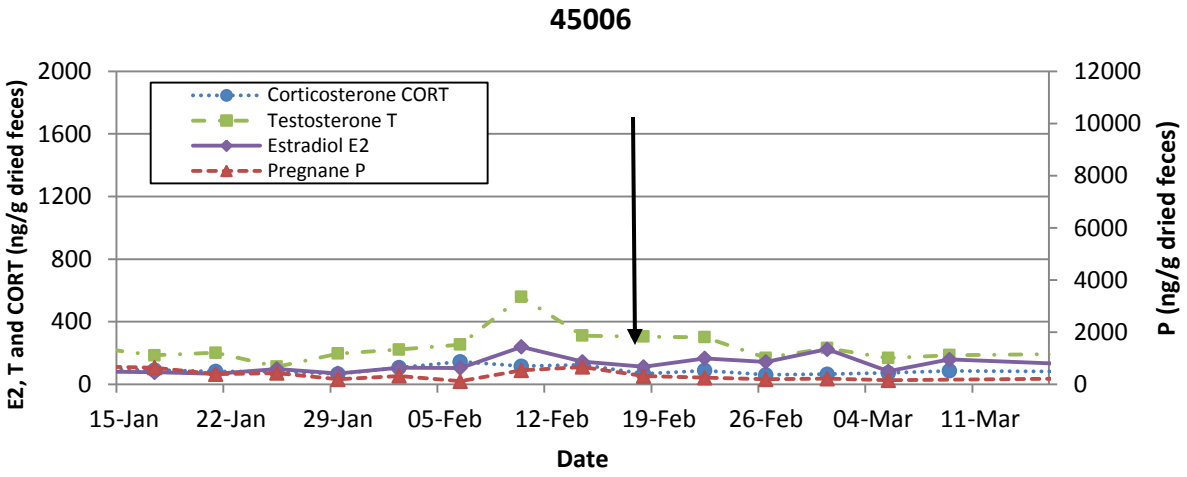
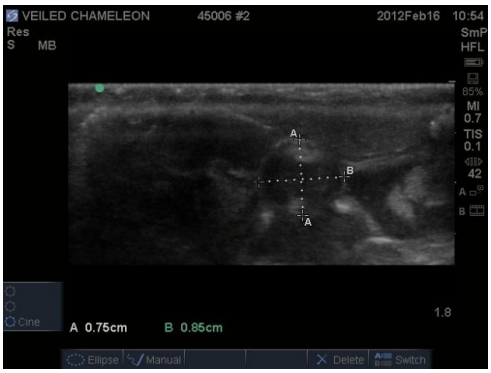


Mar 22, 2012

Measurements	16-Feb-12	23-Feb-12	8-Mar-12	22-Mar-12
Length	0.91	0.93	0.92	1.11
Width	0.77	0.83	0.88	0.83
L:W	1.18	1.12	1.05	1.34

B1





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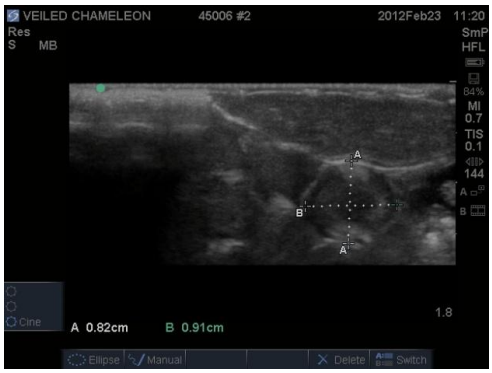

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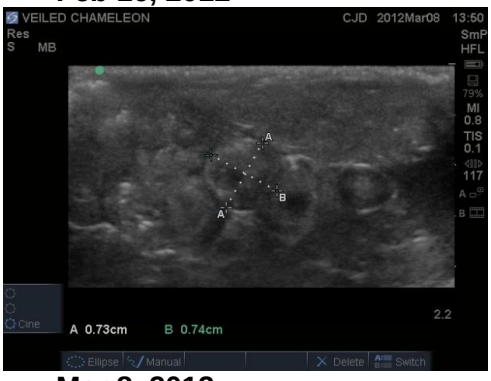

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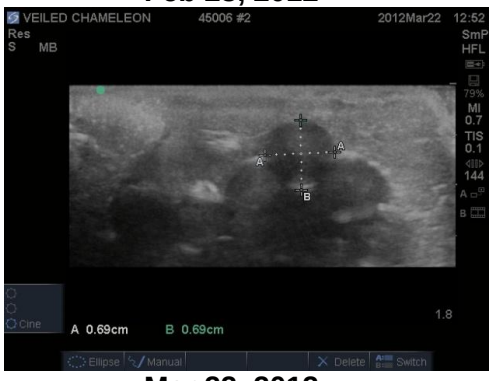
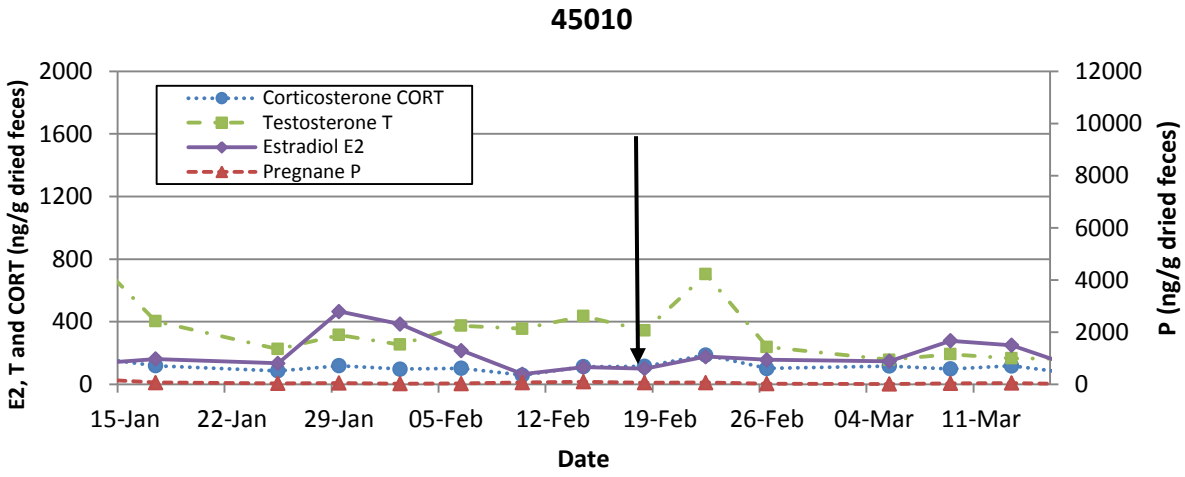


Mar 22, 2012

Measurements	16-Feb-12	23-Feb-12	8-Mar-12	22-Mar-12
Length	0.85	0.91	0.75	0.63
Width	0.8	0.76	0.72	0.57
L:W	1.06	1.20	1.04	1.11

B2



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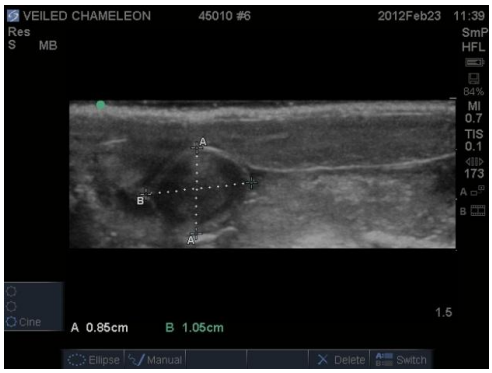

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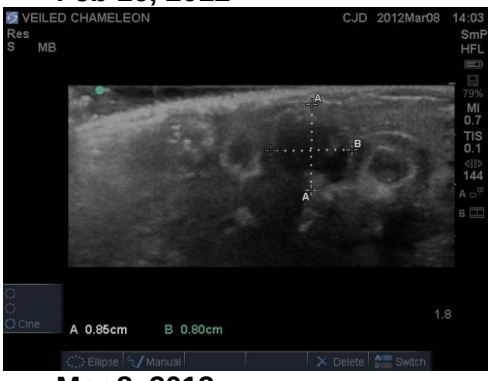

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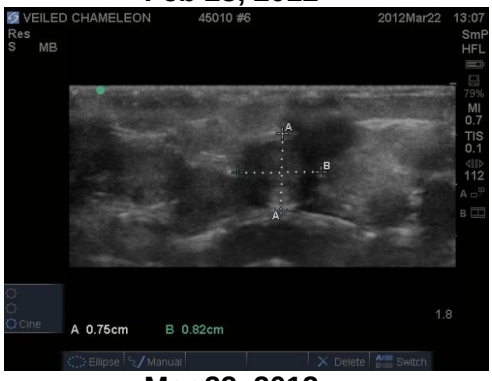
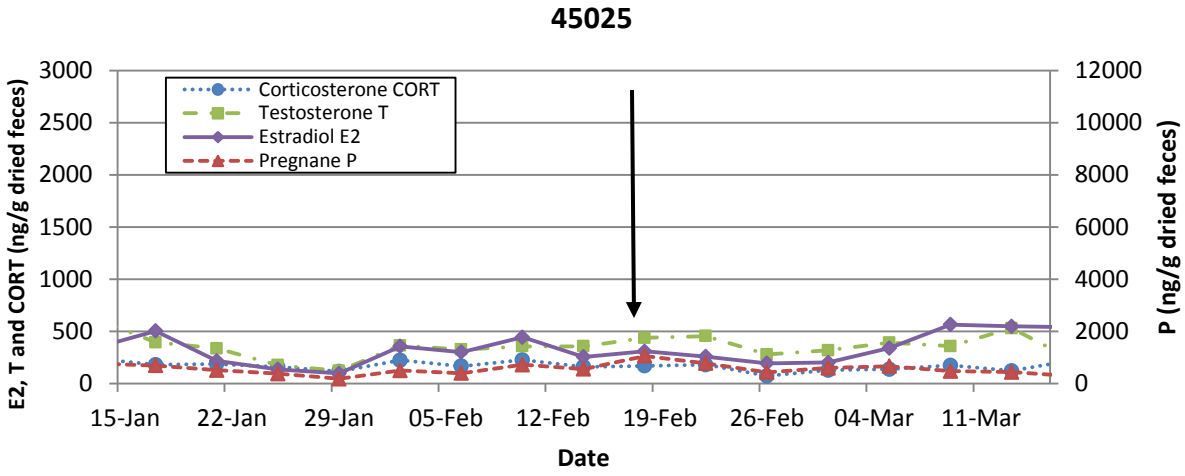
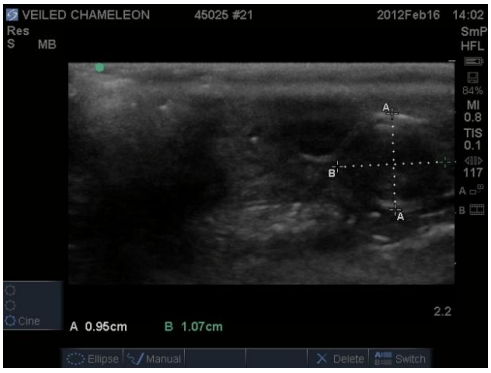


Mar 22, 2012

Measurement	16-Feb-12	23-Feb-12	8-Mar-12	22-Mar-12
Length	0.92	1.03	0.84	0.8
Width	0.92	0.77	0.79	0.76
L:W	1	1.34	1.06	1.05

C1





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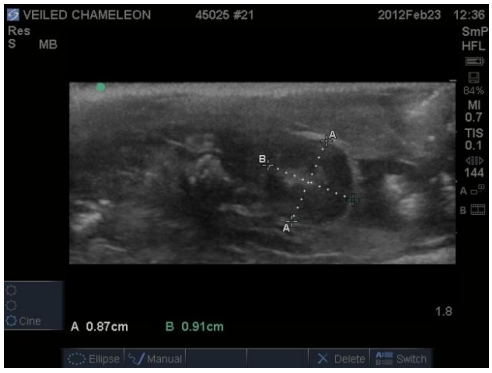

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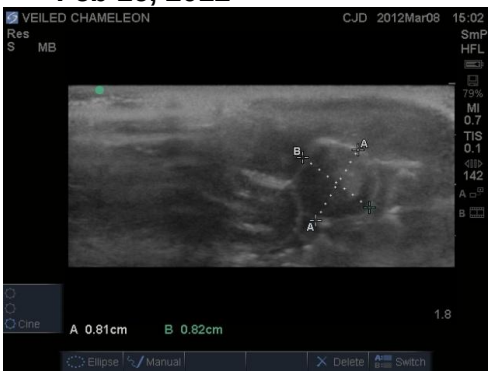

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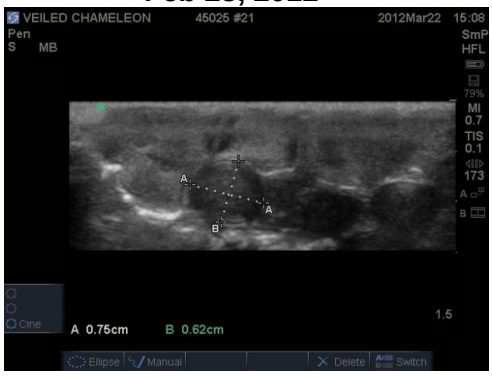
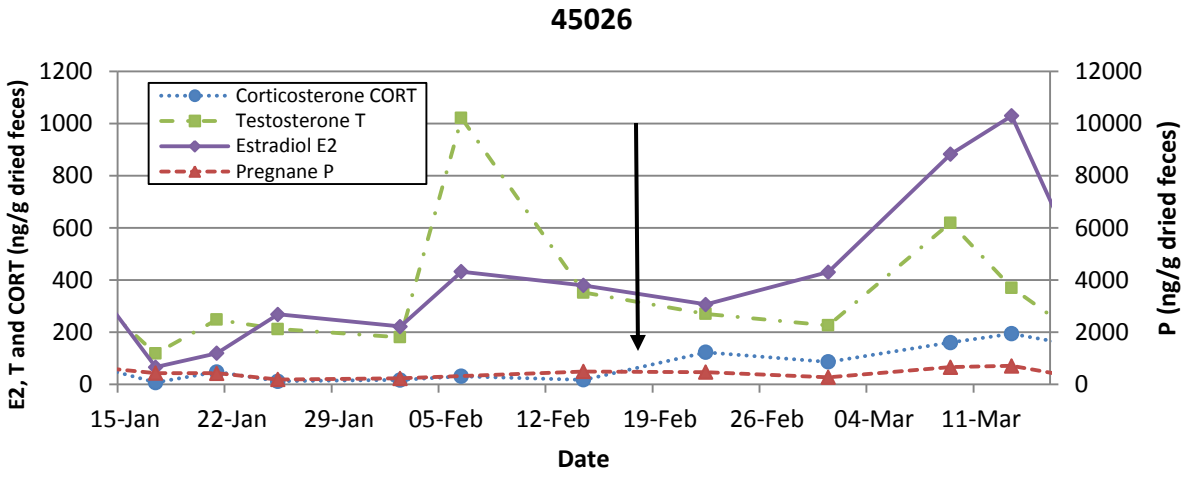
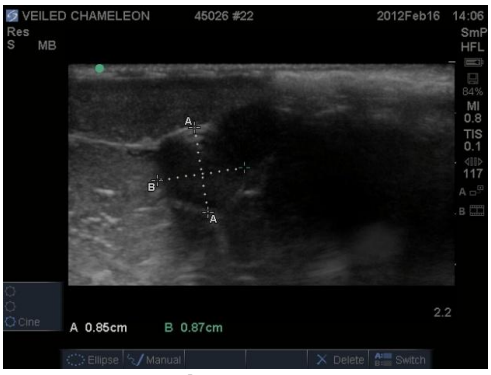


Mar 22, 2012

Measurements	16-Feb-12	23-Feb-12	8-Mar-12	22-Mar-12
Length	0.96	0.97	0.88	0.73
Width	0.86	0.83	0.81	0.61
L:W	1.12	1.17	1.09	1.20

C2





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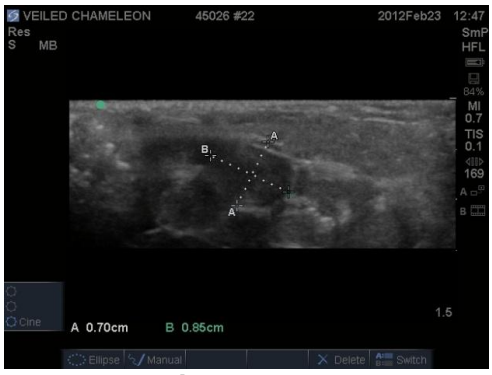

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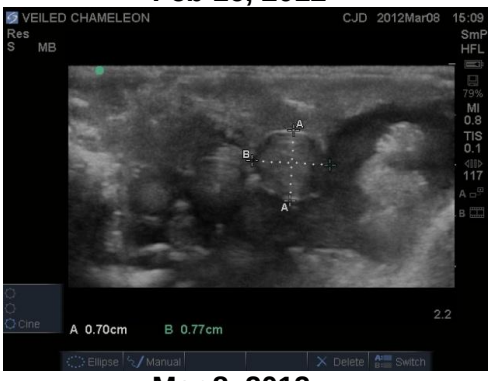
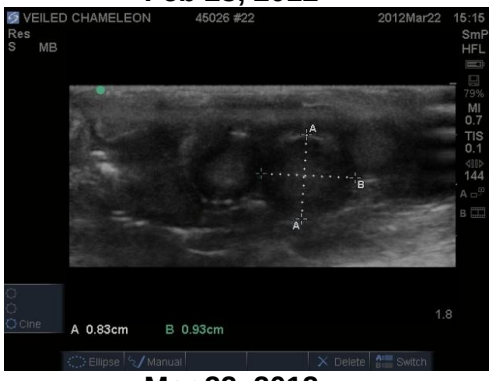


Mar 8, 2012



Mar 22, 2012

Measurements	16-Feb-12	23-Feb-12	8-Mar-12	22-Mar-12
Length	0.78	0.75	0.77	0.9
Width	0.78	0.56	0.63	0.81
Length:Width	1	1.34	1.22	1.11

Fecal hormone patterns of E2, CORT, T and P, ultrasound images, and follicle measurements

for two animals in each treatment group (A, B, and C) of the ovulation induction trial; 45005 and 45017 received chicken GnRH II (A1 and A2), 45006 & 45010 received a combination of progesterone/PGF2α (B1 and B2), and 45025 & 45026 did not receive any treatment (C1 and C2). Arrows indicate date of treatment. Solid, diamond (♦) = E2; dash-dotted, square (■) = T; dashed, triangle (▲) = P; round dotted, circle (●) = CORT.

Feed restriction

Fecal hormone metabolite analyses were not run on samples produced during the 4 week trial period. This was due to the fact that the animals were producing fewer samples (an obvious side effect of reducing food consumption); so gut transit time would have been affected, and as a result, hormone metabolite accumulation in the samples would be misrepresentative of plasma levels. Therefore, we were unable to verify if the dose of progesterone used was sufficient to cause significant elevations in systemic hormone levels, and whether the reduction in feed elicited a stress response in the animals.

At the time of the feed restriction trial it was not possible to distinguish “new” and “old” follicles within the reproductive tract of animals on ultrasound images. Therefore, follicle SA measurements may have been taken from different batches of follicles during the same or subsequent ultrasound exams.

Of the animals on the restricted feeding schedule, 4/7 animals in each of the treatment groups (progesterone, saline, and no treatment) had an overall decrease in follicle SA. One out three animals kept on the regular feeding schedule and diet had a decrease in SA. Increases and decreases in follicle SA did not show a consistent relationship with weight gain or loss, respectively (i.e. animals that gained weight did not always have an increase in follicle SA and vice-versa). There was no apparent relationship between cycle stage and increases or decreases in follicle SA (i.e. decreases in follicle SA occurred in animals that were entering the early to mid vitellogenic phases, and increases in SA occurred in animals that were in atretic or pre-vitellogenic stages).

The results of the feed restriction trial, including averages for overall weight loss (in grams), and relative weight loss (in %), adjusted for initial weight are presented in Table 3.1.

Five out of seven animals in the first group (progesterone), and 7/7 for the groups receiving saline alone, or no treatment had an overall decrease in weight, and all of the animals that were kept on the regular feeding schedule and diet (n=3) gained weight over the four week study period.

Analysis of covariance indicated that the effect of initial weight was very significant, regardless of treatment ($p<0.0001$). ANOVA revealed that feed restriction did not have a significant effect on weight change when compared to animals in the regular feed group ($p>0.60$), and no significant differences were found between treatment groups of feed restricted animals (progesterone, saline, and no treatment) ($p>0.25$).

Table 3.1 Feed restriction trial weight changes

Diet	Treatment	Weight change (g)	Relative weight change (%)
Feed restriction (n = 21)	Progesterone (n=7)	-9.42 (-14.24, -4.6)	-7.24 (-10.95, -5.54)
	Saline (n=7)	-6.52 (-10.93, -2.1)	-0.05 (-0.08, -0.02)
	None (n=7)	-3.15 (-12.83, 6.53)	-0.02 (-0.10, 0.05)
Regular feed (n=3)	None (n=3)	-7.42 (-12.33, -2.52)	-0.06 (-0.09, -0.02)

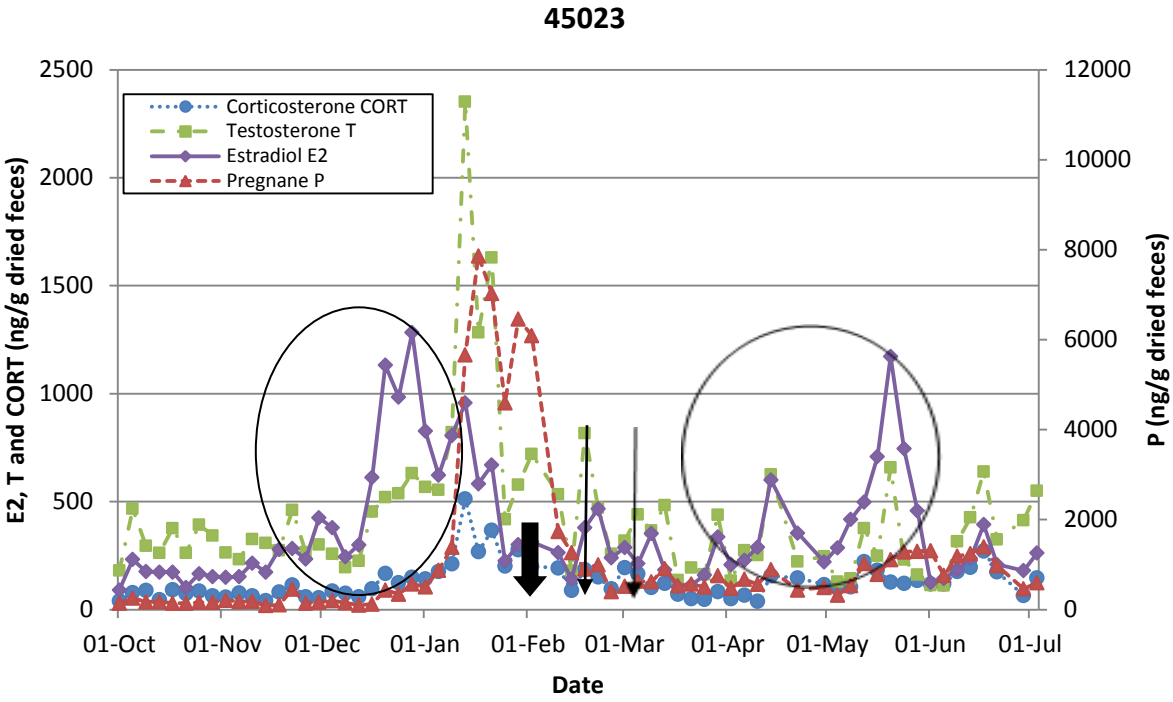
Calculated averages for change in weight (in g), relative change in weight (in %), adjusted for mean initial weight for the feed restriction trial groups (feed restriction with progesterone, saline, and no treatment; and regular feed with no treatment).

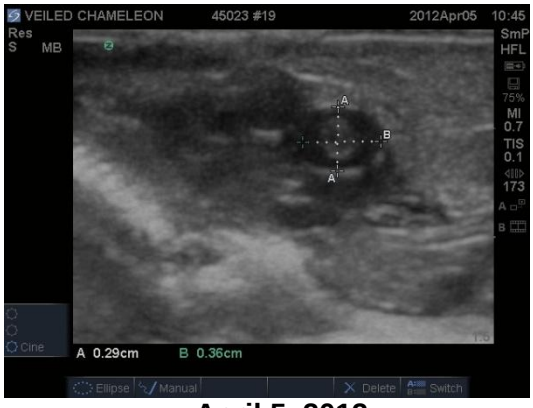
Contraception

Ultrasonography revealed the presence of growing follicles in the weeks following contraceptive treatment in 2/4 animals (ultrasounds discontinued after August 16, 2012), (Figure 3.2). Additionally, fecal reproductive hormone patterns indicated that there was no delay in the initiation of vitellogenesis, as evidenced by E2 levels beginning to increase within 6-8 weeks of treatment in all of the animals (4/4) (Figure 3.2).

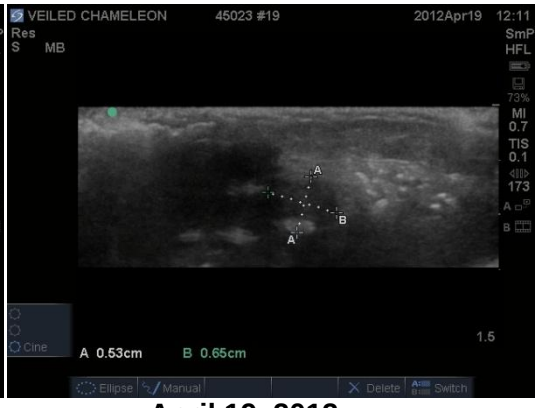
Figure 3.3 Response of female veiled chameleons to contraceptive treatment

A





April 5, 2012



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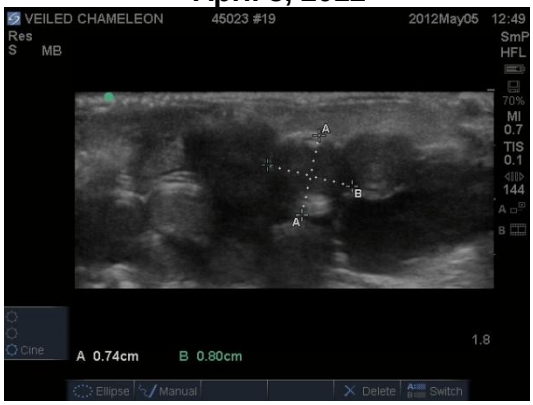
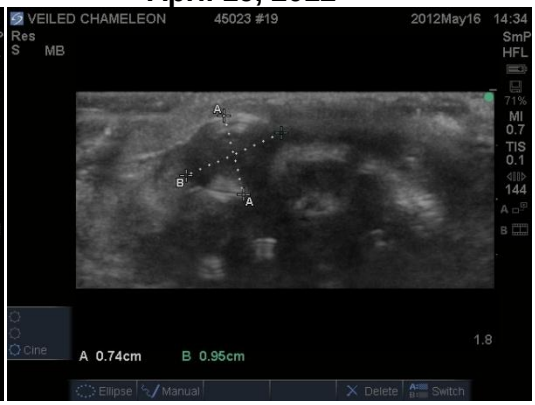


May 5, 2012



May 16, 2012

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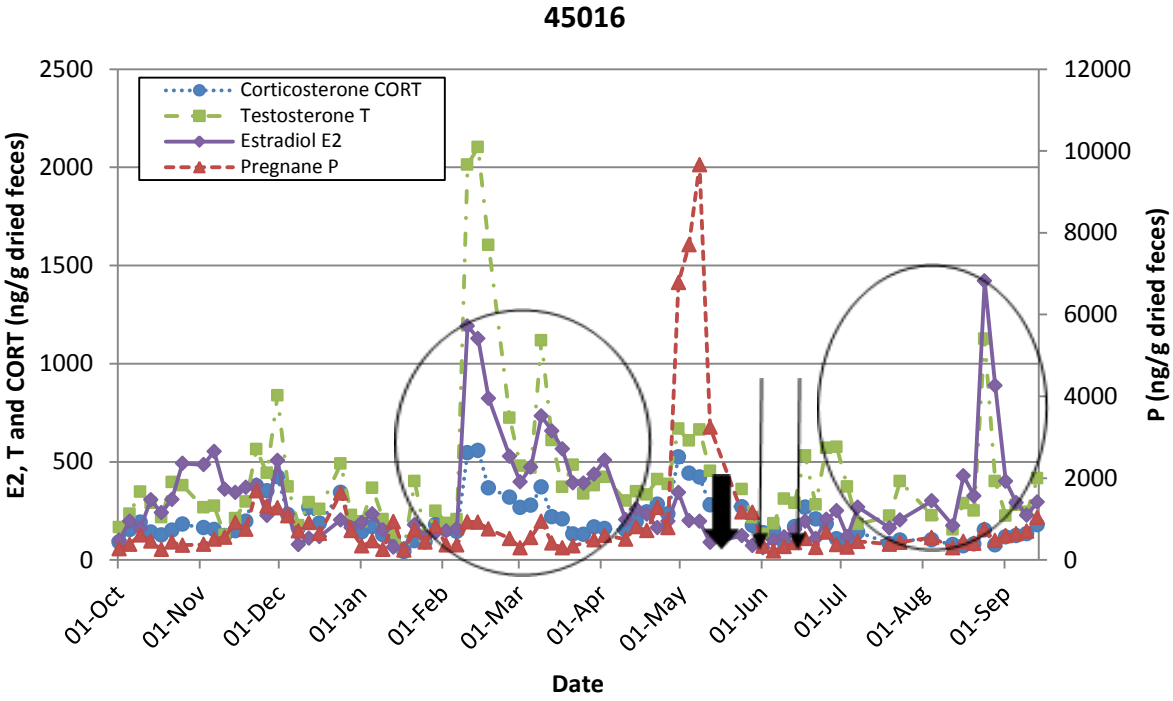
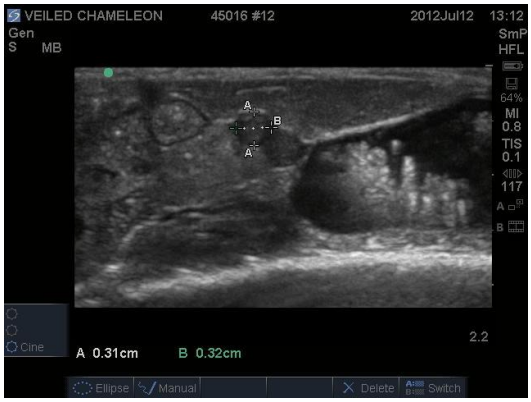
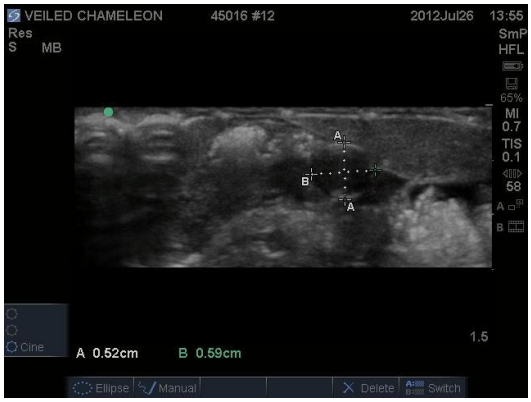




July 12, 2012



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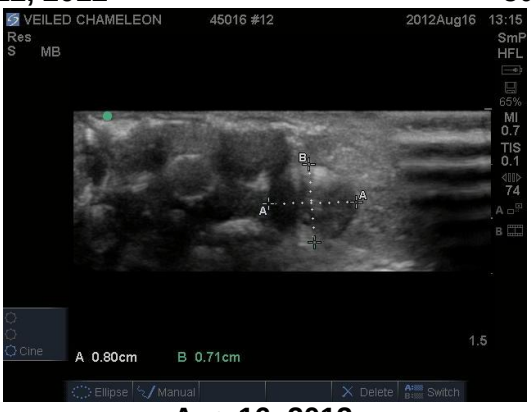
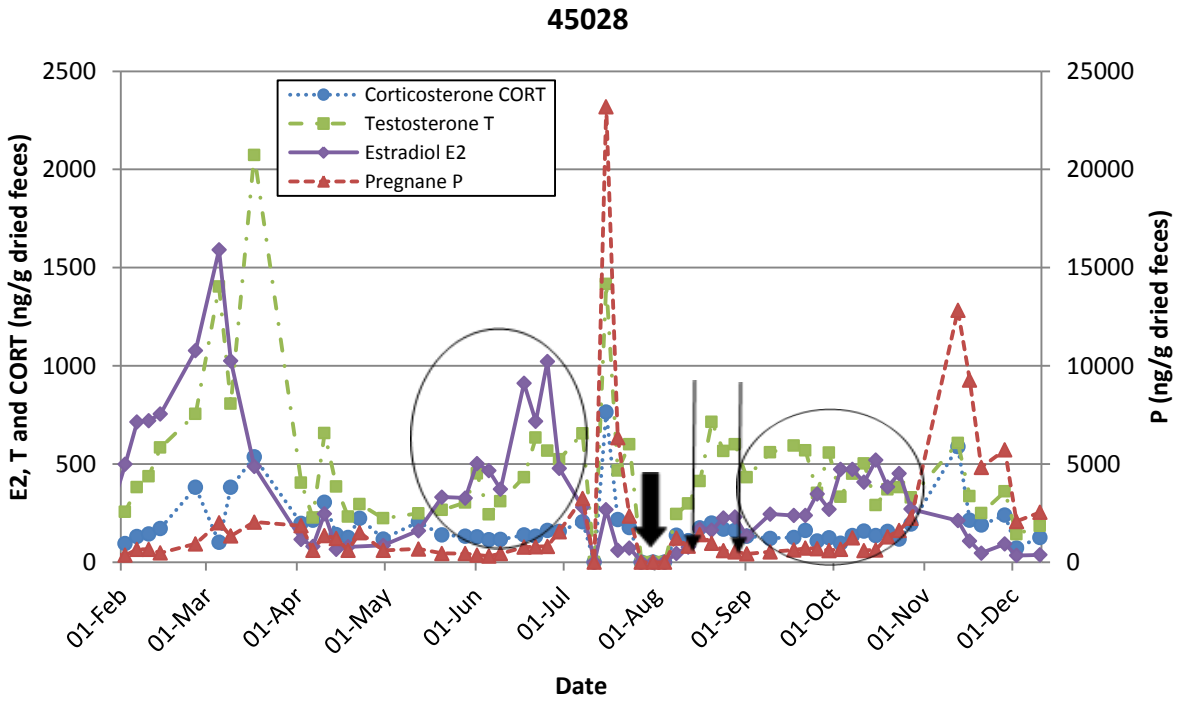
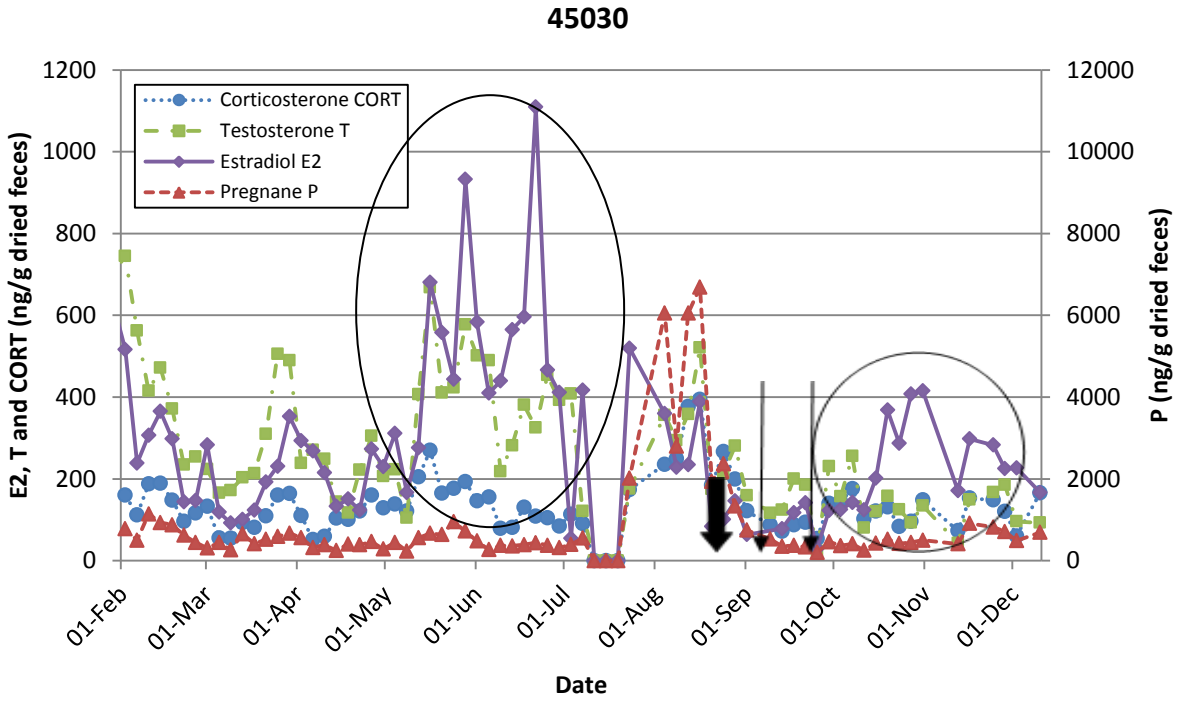


Aug 16, 2012

C



D



Reproductive and adrenal fecal hormone metabolite patterns of E2, CORT, T and P plotted

against time for four female veiled chameleons (45023; A, 45016; B, 45028; C & 45030; D) that received contraceptive treatment following oviposition. Ultrasound images showing vitellogenic growth of follicles over time, following contraceptive treatment for two animals (45023 & 45026) is provided. Periods of vitellogenesis circled; dates of oviposition indicated by thick arrow; and dates of contraceptive administration indicated by thin arrows. Solid, diamond (♦) = E2; dash-dotted, square (■) = T; dashed, triangle (▲) = P; round dotted, circle (●) = CORT.

Biological data

Upon necropsy many of the animals had two distinct batches of follicles (differentiation based on colouration, vascularity, and plumpness; see chapter 1 for full description and images) present on the ovaries. This confirmed the diagnosis of follicular stasis in the majority of the animals. Dates of oviposition were February 3, 2012 (45023), May 15, 2012 (45016), July 28, 2012 (45028) and August 20, 2012 (45030).

DISCUSSION

The specific mechanism and trigger for ovulation in reptiles is not well understood. Only one gonadotropin with mammalian FSH-like properties has been identified in lizards and snakes to date; no LH-like gonadotropin has been isolated (Licht, 1984). Thus, the role of FSH may have additional properties than just aiding in follicle maturation and vitellogenesis; it may also be involved in the process of ovulation (Licht, 1970; Licht, 1984; Callard, 1972a). A few studies have investigated the effectiveness of exogenous doses of gonadotropins to induce ovulation in different species of lizards. Jones et al. (1988) found that porcine FSH successfully induced ovulation in anole lizards (*A. carolinensis*) with mature follicles (>5mm). Similarly, Licht (1970) studied the effects of ovine FSH and LH in *A. carolinensis, U. stansburia*, and *S. occidentalis* and found that FSH successfully induced ovulation, but no synergistic effects were found when LH was used in combination, and LH alone did not have any effect. Purified turtle FSH and LH induced ovulation in *A. carolinensis*, but FSH was considerably more potent (Licht and Crews; 1975). Shanbag and Prasad (1993) induced ovulation in *C. versicolor* using serum gonadotropin (gonadotrophan-FSH). No prior research has been done on the use of GnRH for ovulation induction in reptiles. However, based on the fact that GnRH causes the downstream release of

FSH and LH in mammals, and FSH in reptiles (Licht, 1984), the current study examined the use of GnRH as opposed to FSH.

No studies have examined the use of combinations of P and PGF2α analogues at inducing ovulation in lizards, nor the impact on ovarian steroidogenesis. In a few species of reptiles, there is a decrease in estrogen and increase in testosterone and progesterone prior to ovulation. This has been documented in veiled chameleons (Kumrrow et al., 2010c), bearded dragons; *P. barbata* (Amey and Whittier, 2000), *A. carolinensis* (McNicol and Crews, 1979), blue tongued skinks; *T. nigrolutea* (Edwards and Jones, 2001), among others. The trigger for ovulation in these species is not well understood, but it has been suggested that progesterone is a key factor. Kummrow et al. (2010c) speculate that it is the ratio of decreasing estrogen:progesterone that stimulates ovulation in *C. calyptratus*, and Amey and Whittier (2000) suggest that the main functions of progesterone in oviparous species (such as *P. barbata*) are the stimulation of follicle maturation and ovulation, and preparation of the oviduct for this event.

Studies in amphibians have shown that progesterone is capable of inducing ovulation *in vitro* from ovaries of *R. cyanophlyctis* (Ramaswami and Lakshman, 1958) and *R. pipiens* (Wright, 1961 & 1971; Edgren and Carter, 1963). However, those studies were conducted using portions of excised ovaries of varying masses, and doses ranging from 5 µg/10ml – 300 µg/ml, so it is not possible to make direct comparisons with the current study. However, in the studies where a range of doses were used, the authors report that higher concentrations of progesterone were less effective than lower doses, above a minimum threshold. This implies that ovulation is dose-dependent, so further research investigating the effects of varying doses of progesterone on the ovaries (and ovulation rates) in both healthy and egg-bound reptiles is warranted.

. Prostaglandins (PGs) play an important role in preparing the follicle for expulsion from the ovary prior to ovulation in many species of mammals and fish, based on the fact that administration of aspirin or indomethacin (PG synthesis inhibitors) blocks ovulation (see Armstrong, 1981 for review). Furthermore, Criscuolo-Urbinati et al. (2012) determined that administration of PGF significant improved ovulation rates in the teleost fish, *Piaractus mesopotamicus* in a population with low rates of spawning. In addition, Jones et al. (1990) found that indomethacin blocked FSH-induced ovulation in *A. carolinensis*, and suggested that PGs may mediate or permit gonadotropin influence on pre-ovulatory follicles. Therefore, it is possible

that both progesterone and PGs are necessary during the pre-/ovulatory process but further research into the use of these drugs is needed to confirm this theory.

The results of the ovulation induction trial indicated that neither of treatments (cGnRH-II or P/ PGF2α) were successful in animals exhibiting follicular stasis. There are a few possible explanations: 1) the doses of hormones selected were not high enough to cause an increase in serum levels that would induce physiological events; 2) the route of administration (i.m.) prohibited absorption of the drugs; or 3) ovaries with atretic follicles no longer contain the receptors to bind ovulation-inducing hormones. Reproductive hormone profiles did not show any distinct increases in any of the sex steroids following injection of GnRH or progesterone analogues, which suggests the first or second explanations are likely. However, Kummrow et al. (2010b) found that 3/6 female veiled chameleons injected with c GnRH II at a dose of 100 µg/kg i.m. displayed elevated levels of E2 following administration, while the remainder showed no response. They report that the animals used for the trial were in different stages of their reproductive cycles, which could have affected the animals' responsiveness to GnRH, but those stages were not specified. Therefore, it is not possible to eliminate cycle stage as a confounding factor affecting response to c GnRH II (explanation #3).

The results of the feed restriction trial indicate that a reduction in food did not have a significant effect on body weight change when compared to animals on the regular feeding schedule when adjusted for inititial weight. Additionally, there was no effect of treatment (progesterone, saline or no treatment) on weight change. However, the high number of animals that lost weight in the feed restriction group (19/21) suggests that the feed restriction induced an energy-deficient state, based on the fact that all of the animals on the regular diet (3/3) gained weight. The results do not confirm whether feed restriction and subsequent weight loss were directly related to changes in follicle surface area. Additionally, the changes in follicle size were not related to the stage of the reproductive cycle the animals were in at the start of the trial. Since the criteria to distinguish new and old follicles was determined following termination of the study, conscious efforts to measure both types of follicles at the start and end of the study were not made. Therefore, increases or decreases to follicle surface area may not have been an accurate reflection of which batch of follicles (if either) were changing in size.

Progesterone was selected for treatment in order to attempt to imitate the gravid state, during which high levels of progesterone are secreted from the corpora lutea formed following

ovulation (Callard and Ho, 1980; Callard et al., 1992; Kummrow et al., 2010c; Shanbag et al., 2002). In addition, Bragdon (1952) suggests that granulosa cells responsible for yolk phagocytosis during atresia secrete progesterone as a by-product. Therefore, we hypothesized that artificially raised endogenous levels of progesterone over several days, would stimulate the initiation of yolk reabsorption from follicles that failed to ovulate. Additionally, high levels of progesterone would create a negative feedback loop on estrogen secretion, which would presumably decrease the amount of FSH being secreted by the hypothalamus and vitellogenin being sequestered from the liver. Callard et al. (1972) found that progesterone injections given during vitellogenesis inhibited follicular growth and caused some follicular atresia in female blue spiny lizards (*S. cyanogenys*). However, the results were inconclusive since increases or decreases in follicle size from different batches could not be confirmed.

Currently no research on the use of GnRH to alter follicular development in reptiles has been published, although several studies have been done in mammals and a couple in birds. For example; Alkis et al. (2011) found that conception rates were 0% compared to 100% in rats receiving deslorelin (a GnRH agonist) administered by way of a subcutaneous controlled release implant, and no treatment, respectively. Millam and Finney (1994) found that Lupron Depot administered intramuscularly (reversibly) delayed egg-laying in cockatiels (*Nymphicus hollandicus*) by 12-19 days (higher doses having a longer lasting effect), compared to animals that did not receive treatment. In contrast, a study by Constantini et al. (2009) showed that a single subcutaneous administration of GnRH analogue, buserelin acetate increased laying rates and fecal estradiol metabolites in budgerigars (*Melopsittacus undulates*) compared to control groups receiving no treatment. The disparity in results between the two studies could be related to the doses used; in the study by Constantini et al. (2009) they used a dose of 10 µg/kg, whereas Millam and Finney (1994) used doses of 17, 52, or 156 µg/kg, with the most pronounced effects observed at the higher doses. Treatment with low doses of GnRH analogues has a stimulatory effect, inducing a large increase the production and release of FSH and LH from the pituitary gland, and was initially used to treat infertility (Herbert and Trigg, 2005; Schneider et al., 2006). However, a high dose or chronic administration results in a refractory effect, whereby GnRH receptors are down-regulated and there is desensitization of pituitary gonadotropes. Therefore, it is possible that the low dose and volume used in budgerigars was absorbed quickly, eliciting a short-term effect, resulting in stimulation of the ovaries.

Preliminary results of the contraceptive trials indicate that neither contraceptive was

effective at preventing follicular development when administered in the previtellogenic phase. Both of the drugs selected for the trials were depot formulations, designed for steady release over the course of 1 & 3 months (for 3.75 mg Lupron Depot and 50 mg/ml Depo-Provera, respectively) in humans. It is possible that the hormone analogues were not absorbed in large enough quantities, if at all from the muscles, which would inhibit any physiological action they might exert. Studies in reptiles have proven that progesterone has anti-gonadal effects; arresting follicular development, inducing yolk regression, causing decreases in ovarian and oviductal weights, and preventing ovulation. However, delivery method and what stage of development progesterone was administered had durastic impacts on its effectiveness. For example, Callard et al. (1972a) found that subcutaneous injections of progesterone (in sesame oil) given for 28 days, starting during mid-vitellogenesis inhibited ovulation and ovarian growth in *S. cyanogenys*, while injections starting in early vitellogenesis (for 14 days), and intrahypothalamic or subcutaneous implants inserted during late vitellogenesis had no effect on those parameters. However, intrahypothalamic implantation during mid-vitellogenesis inhibited follicular growth and failure to ovulate in the same species (Callard et al., 1972b; Callard and Doolittle, 1973), but implanted subcutaneously or intracerebrally had no effect (Callard and Doolittle, 1973). Therefore, the failure of progesterone to inhibit follicular development in the current study could have been the result of the delivery method, and not the drug itself.

The same forms of GnRH found in birds have been identified in reptiles (cGnRH I and II), therefore it may be possible to extrapolate the effects of GnRH agonist effects seen in birds to reptiles. However, the metabolism of these two groups varies considerably; therefore, the dosage and delivery methods may need to be adjusted for reptiles. The lack of response to either of the drugs used in the current study suggests that there was an issue with absorption, rather than dosage.

CONCLUSIONS

In this study we examined different methods to overcome follicular stasis in a population of female veiled chameleons with a high prevalence of this disorder. Attempts at ovulation induction using GnRH and progesterone/PGF2α analogues were not successful, but further investigation is needed to determine if the treatments failed because the doses used were sub-threshold to elicit an effect, and/or if static follicles no longer contain receptors/are responsive to

ovulation inducing agents. Preliminary data on contraceptive use proved unsuccessful using depot formulations of GnRH and progesterone analogues. Future studies should examine the use of different forms of drug administration and doses are warranted.

Feed restriction proved to be an effective method for inducing an energy-deficient state in the females in the current study, but whether this had an effect on static follicles remains unclear. Further research is necessary to determine if this is an effective treatment for follicular stasis and determine standardized protocols for amount of time and food allowance required to induce the desired changes.

DISCUSSION AND CONCLUSIONS

The main purpose of this study was to develop protocols for the diagnosis and treatment of follicular stasis, and determine the relationship between the adrenal and reproductive axes in female veiled chameleons during ovulatory and non-ovulatory cycles, and in response to dramatic alterations to the external environment.

In chapter 1 we investigated three methods to determine reproductive status; by means of fecal hormone metabolite analysis, behavioural and physical responses to male introductions, and ultrasound imaging of the reproductive tract. Fecal hormone metabolite analysis confirmed the occurrence of non-ovulatory cycles, as evidenced by the absence of the surge (>20-fold above baseline values) of progesterone that follows ovulation, but failed to provide evidence of ongoing follicular stasis since regular cyclical hormones were observed in all animals. Female receptivity; as gauged by behavioural and colour changes in response to being visually exposed to males; did not show any correlation with pre- or post-ovulatory phases of the reproductive cycle, and more specifically between different stages of follicular development. Ultrasound imaging proved to be a valuable tool for distinguishing between pre- and post-ovulatory eggs when comparing consecutive images taken from the same animal over several weeks, and to confirm the occurrence of follicular stasis.

The relationship between the reproductive and adrenal axes was examined in chapter 2. The results indicate a cyclical pattern in corticosterone production which varied with reproductive stage. There were not enough animals to make a statistically significant comparison between non-ovulatory and ovulatory cycles, but of the animals that did ovulate, maximum peak values of fecal corticosterone metabolites were higher in productive complexes.

It could not be determined if dramatic alterations to the external environment elicited a stress response, and subsequently, if that was the cause of the high incidence of follicular stasis in the study population, although it appears unlikely. Future studies comparing adrenal activity between groups maintained in constant versus changing environments is warranted to determine the impact of such events.

The high incidence of follicular stasis in the study population warranted the investigation into possible non-surgical treatments to avoid potential pathologies associated with this

condition. In chapter 3, attempts at inducing ovulation by administration of GnRH or a combination of progesterone and prostaglandin analogues were unsuccessful. It is likely that atretic follicles lack the receptor sights for the hormones that trigger ovulation. A feed restriction trial did not have significant effect on changes in body weight when adjusted for initial weight and compared to animals on the regular feeding schedule. Additionally, it remains unclear whether yolk reabsorption occurred in “new” or “old” follicles on the ovaries, although initial results did not show a consistent relationship between changes in follicle surface area and changes to body weight or stage of the reproductive cycle.

The etiology of follicular stasis remains unclear, but factors such as male presence or absence at different stages of the cycle, constant environmental conditions (i.e. photoperiod, temperature, humidity), and diet have been implicated as possible causes. Future studies investigating the impact of male presence during different stages of the cycle, and differences among females that are courted and/or mated to those that are housed singly should be conducted to determine if males have a significant impact on the reproductive process of a given species. The influence of different diets on egg production; including the size and number of eggs produced, and reabsorption rates of atretic follicles; should be investigated, as this is likely to have a significant effect. Manipulation of light:dark cycles and temperatures at different times of the year could help restore seasonal cues for the onset and termination of reproductive cycles/egg production, although this may not be a feasible option for institutions where animals are on display year-round.

Finally, contraceptives may serve as a method to temporarily block egg production in females when it is not desired. However, further investigation into the effectiveness of different hormone agonists, modes of delivery, and dosage are needed.

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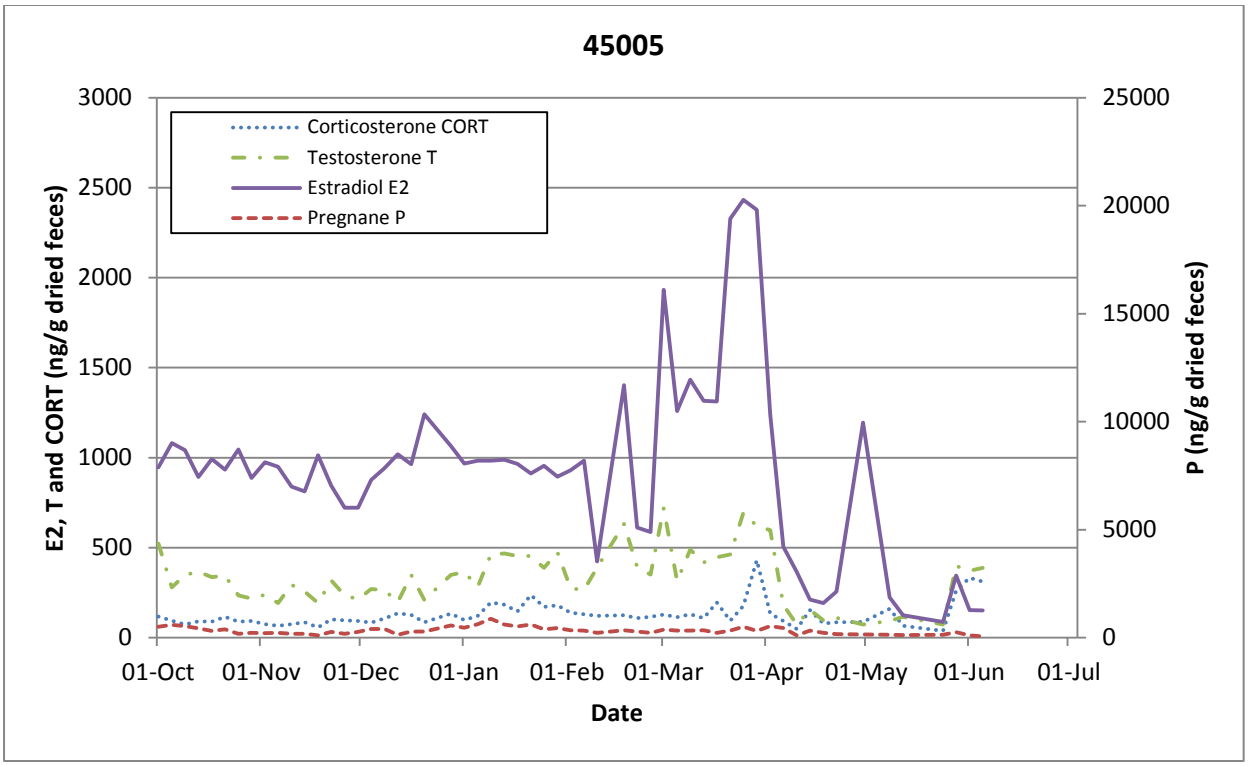
Characterization of follicular stasis in a colony of female veiled chameleons (*Chamaeleo calyptratus*)

SUPPLEMENTAL MATERIAL 1

Fecal hormone patterns and follicular growth curves from individual veiled chameleons: processed raw data

Fecal hormone metabolite patterns (in ng/g dried feces) and follicle growth curves (length and width in cm; surface area in cm^2) for individual animals. Numbers on the top of the graphs are identification numbers of animals. Dates on x-axis are relative to duration of the study (October 2011-July 2012). E2= estradiol, T= testosterone, P= progesterone, CORT = corticosterone.


45005
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
3000
2500
2000
1500
1000
500
0
25000
20000
15000
10000
5000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
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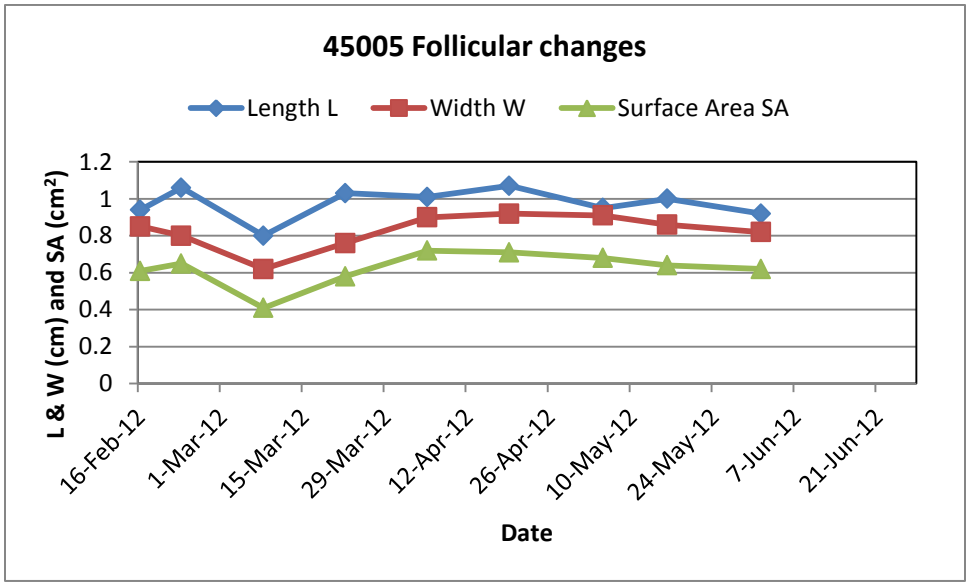
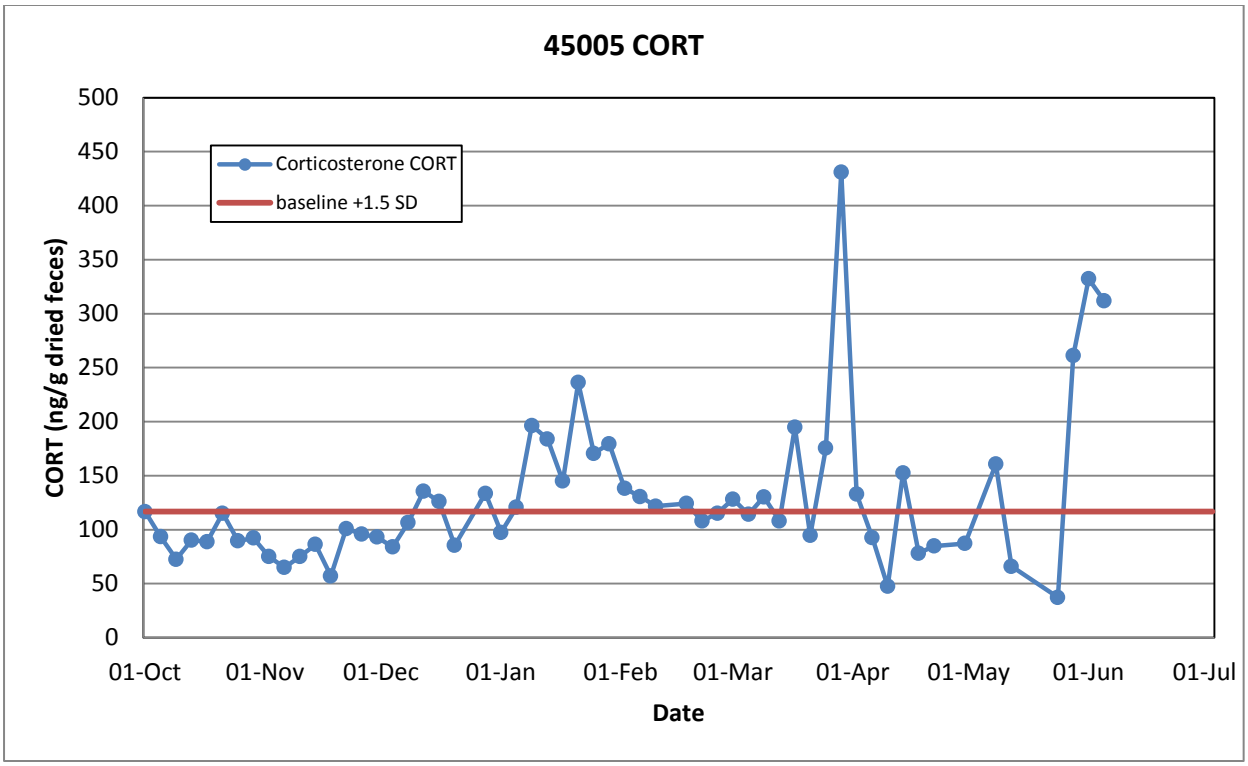
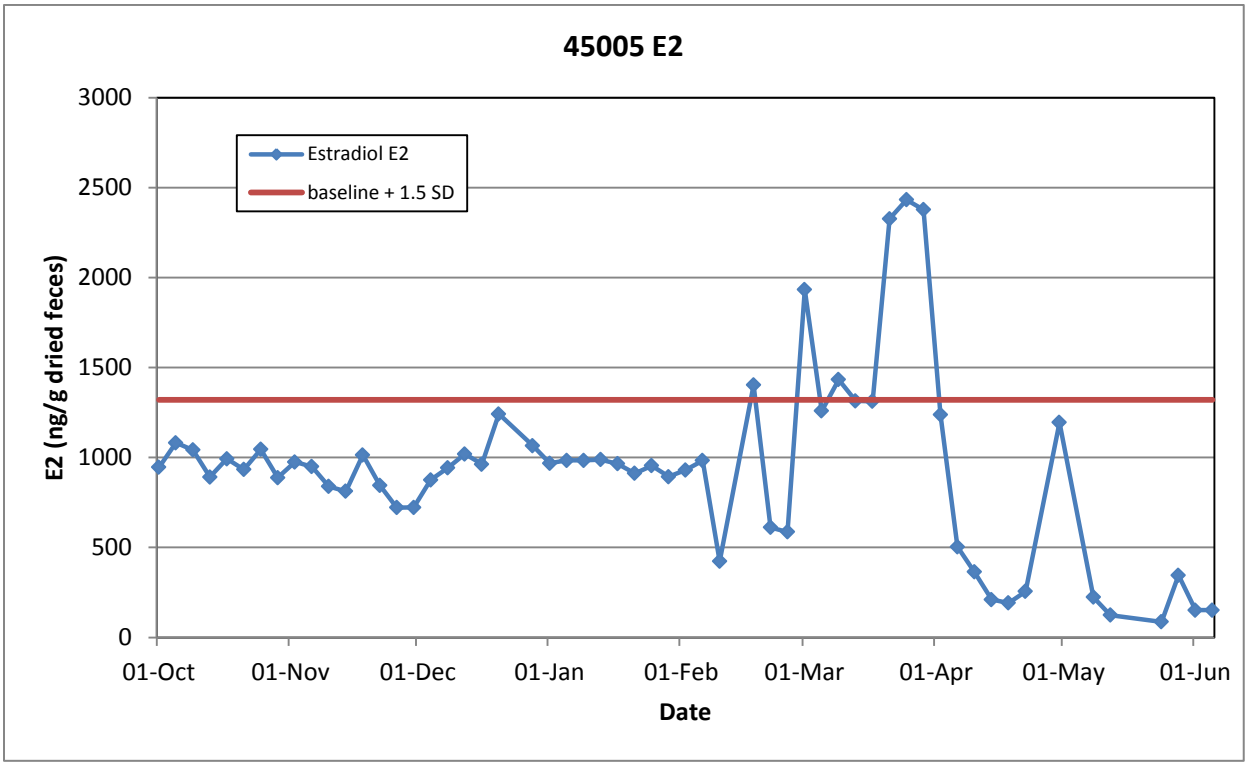
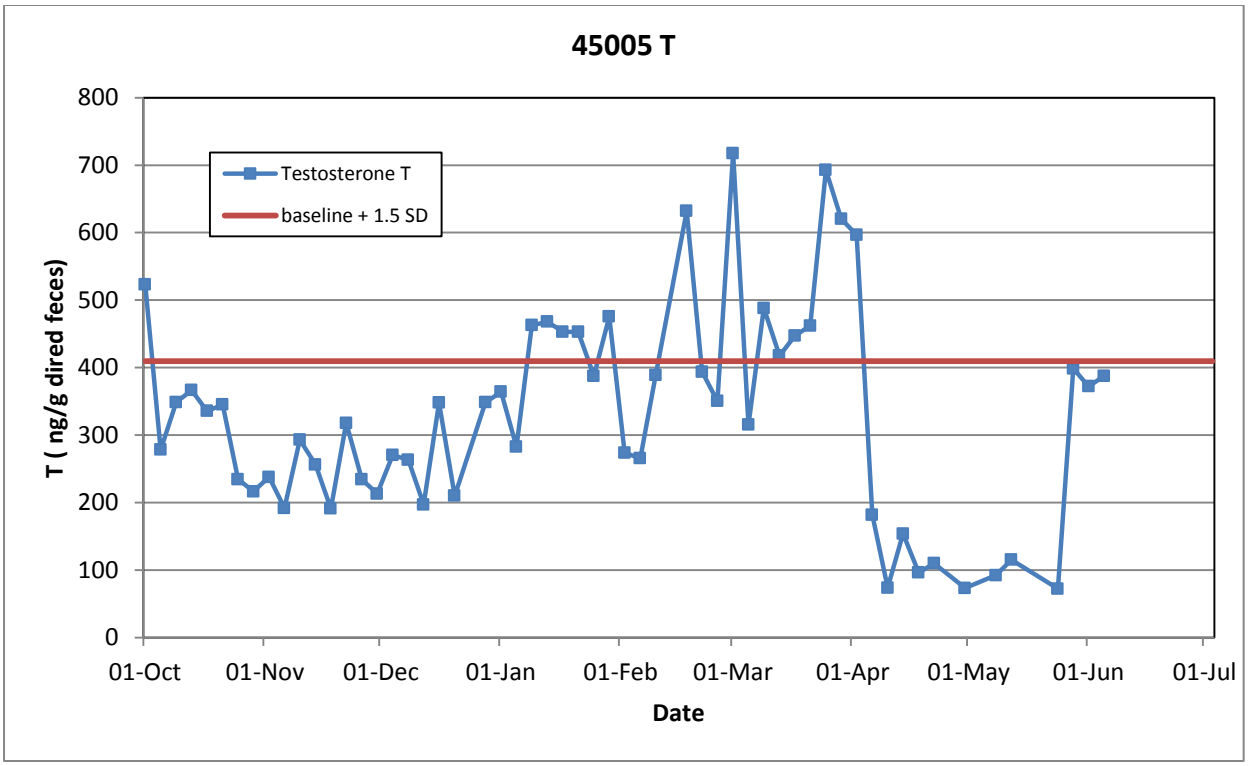
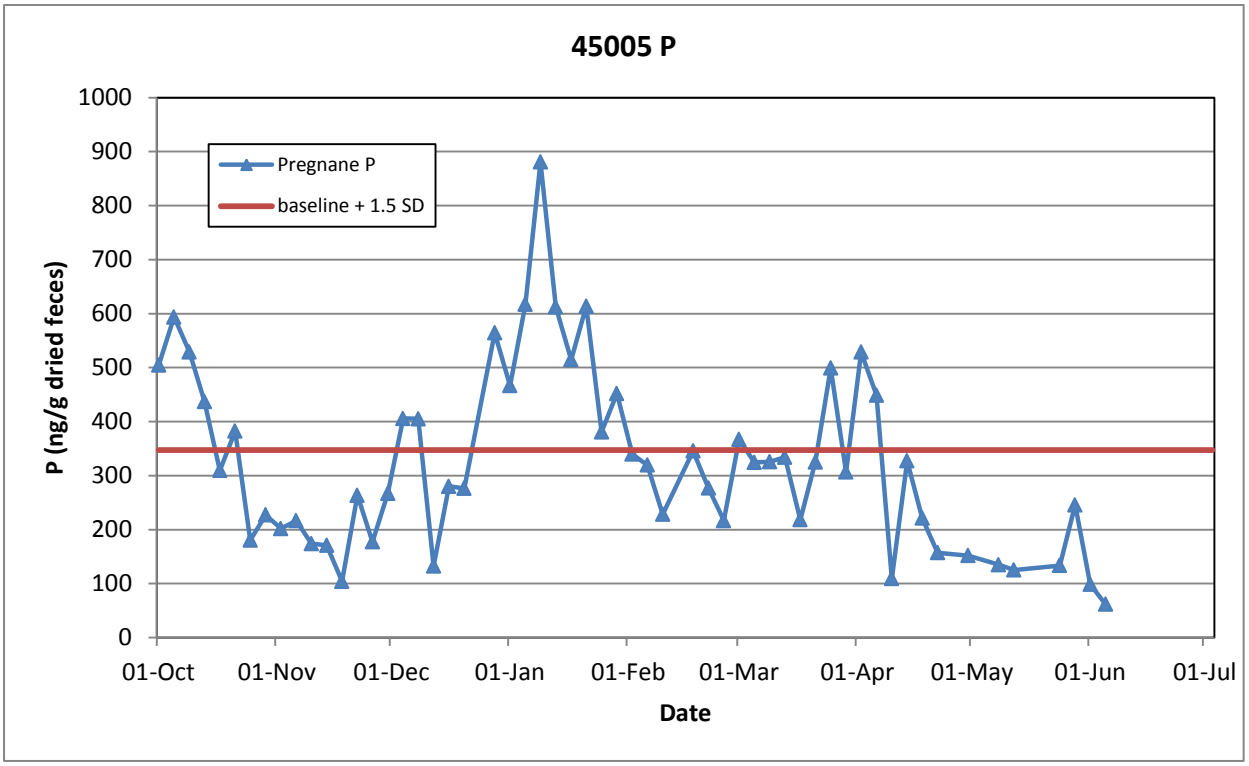
01-May
01-Jun
01-Jul
Date

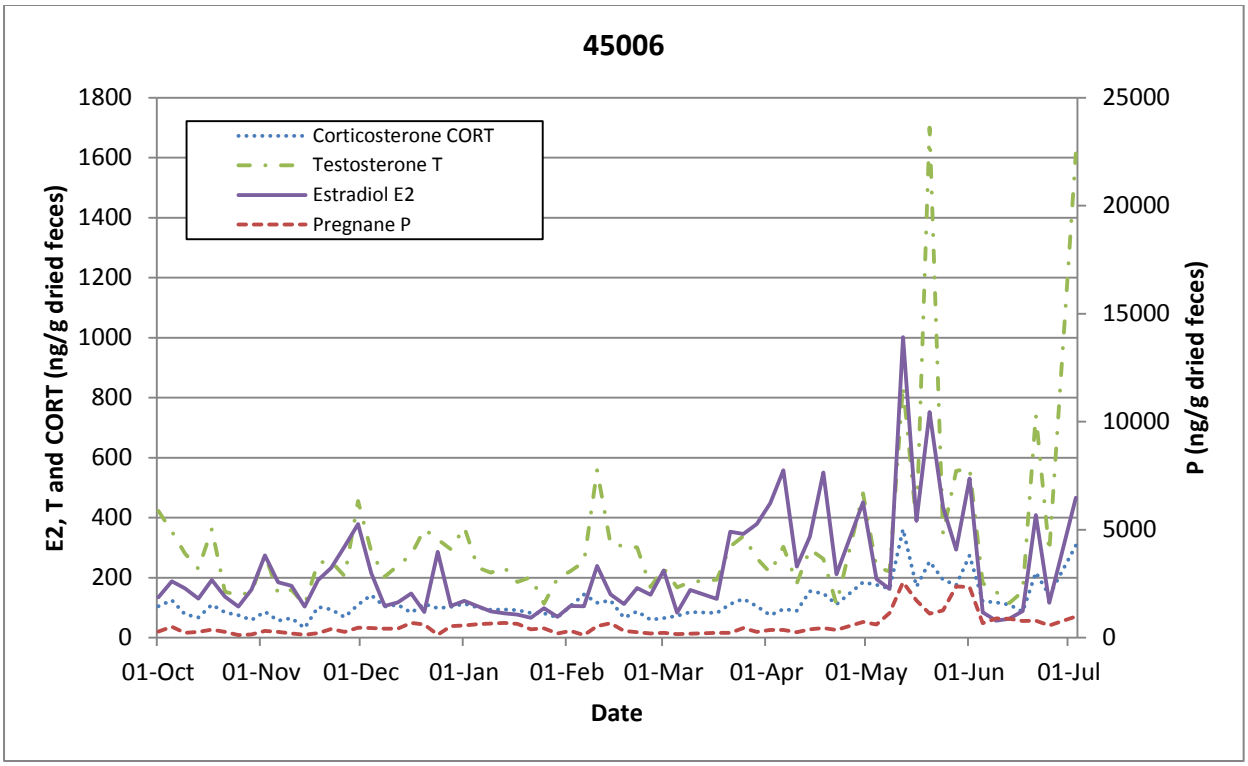

45005 Follicular changes
Length L
Width W
Surface Area SA
L & W (cm) and SA (cm²)
1.2
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

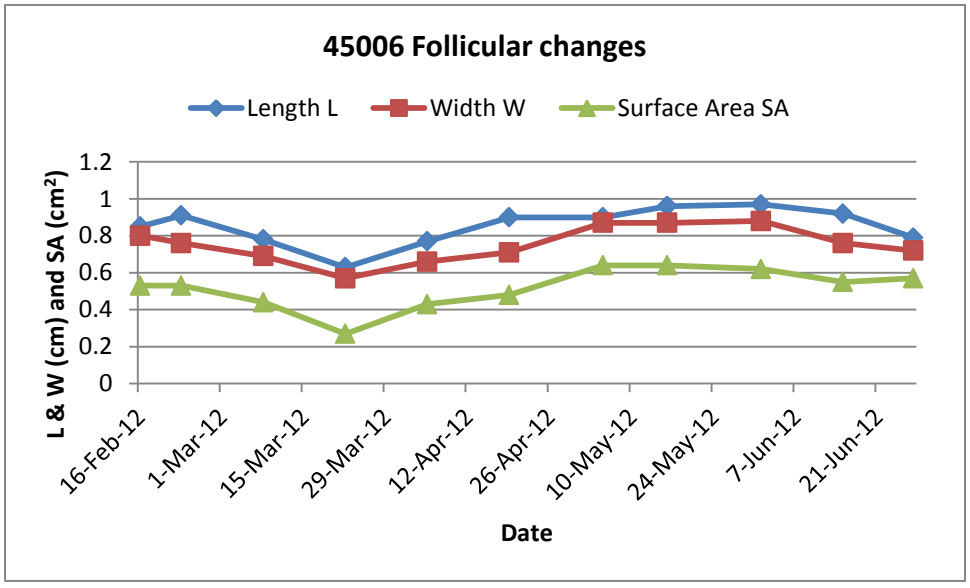

45005 CORT
Corticosterone CORT
baseline +1.5 SD
CORT (ng/g dried feces)
500
450
400
350
300
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

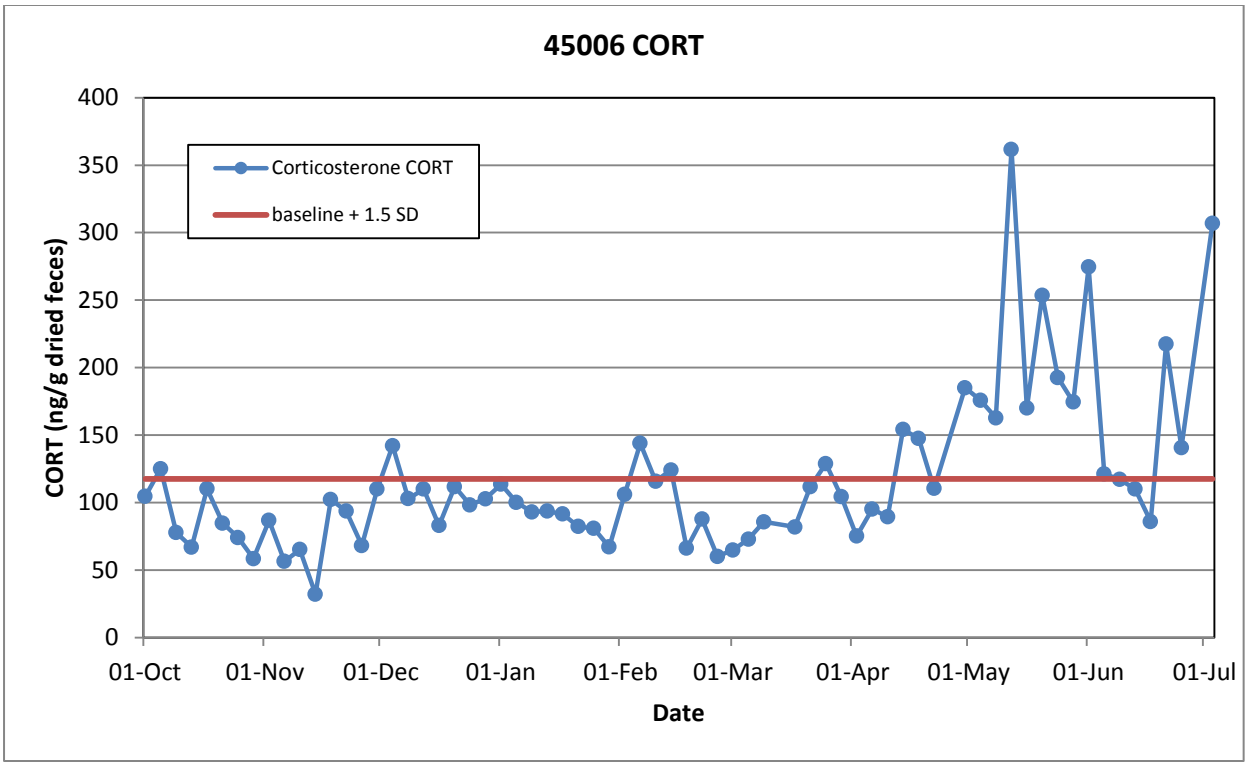

45005 E2
Estradiol E2
baseline + 1.5 SD
E2 (ng/g dried feces)
3000
2500
2000
1500
1000
500
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
Date

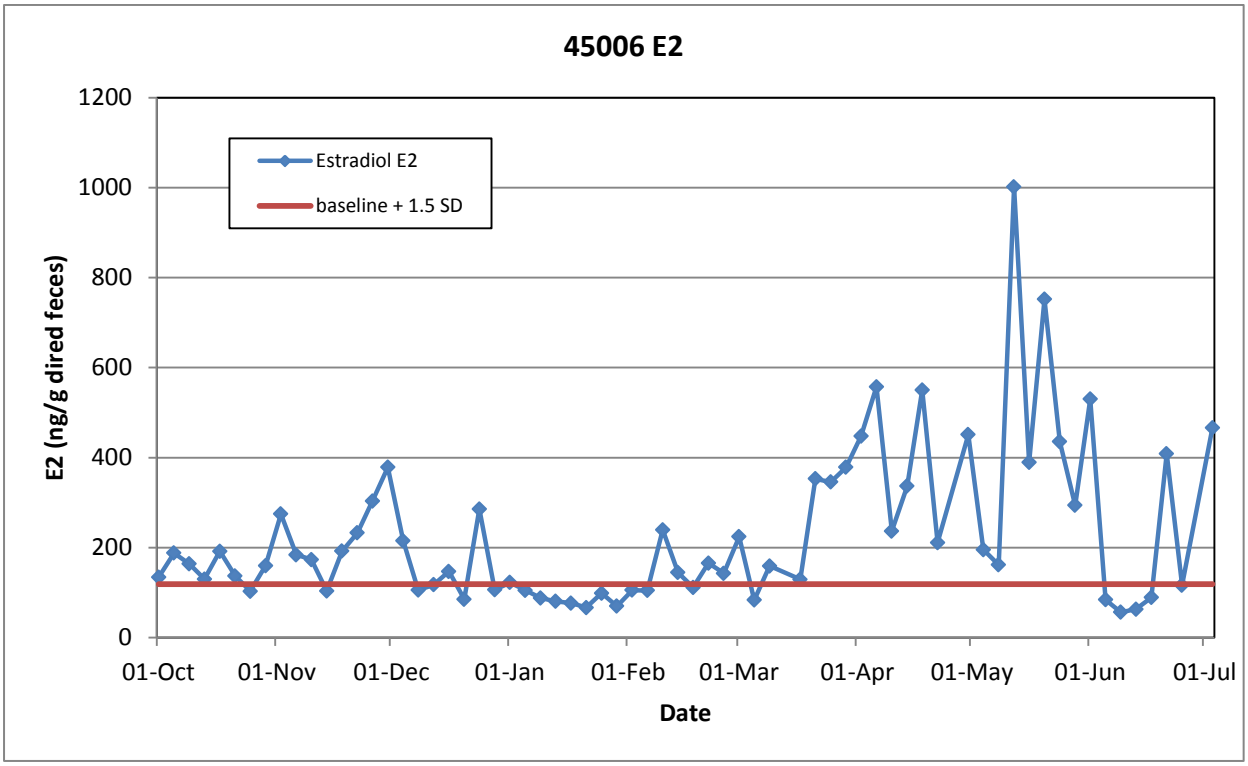

45005 T
Testosterone T
baseline + 1.5 SD
T (ng/g dired feces)
800
700
600
500
400
300
200
100
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

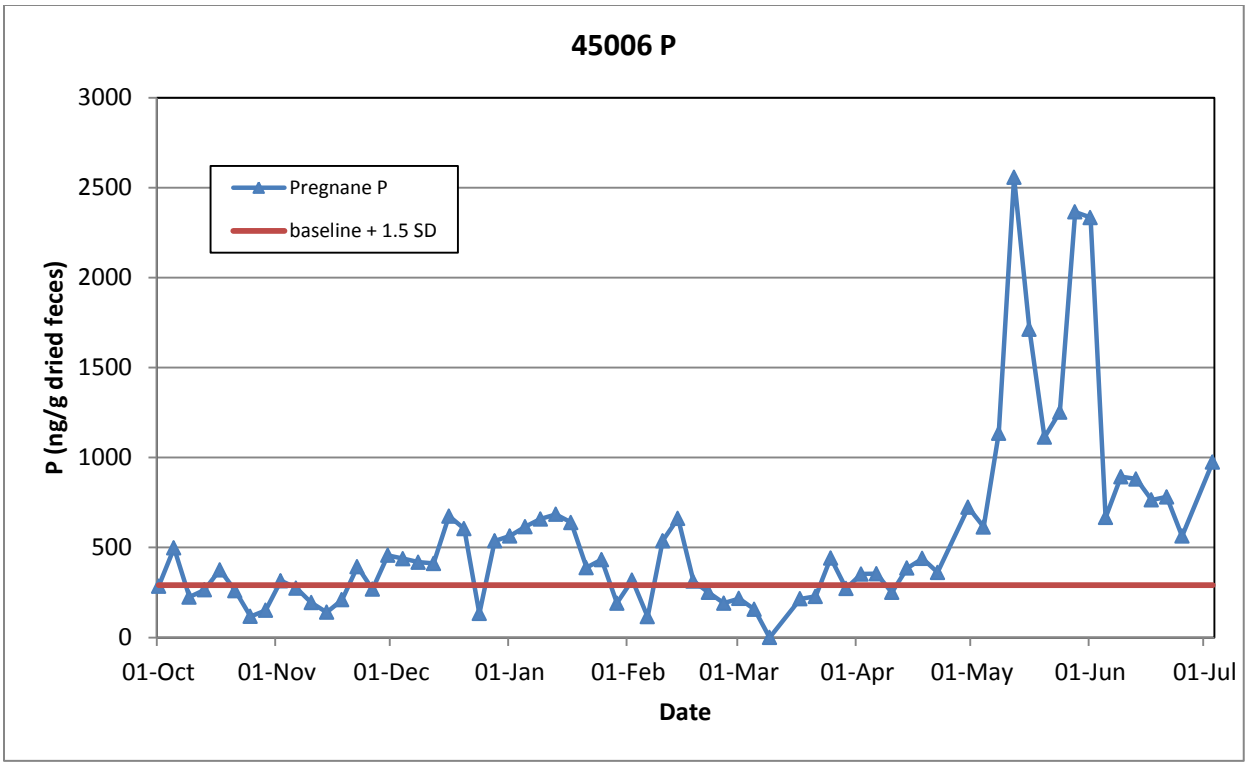

45005 P
Pregnane P
baseline + 1.5 SD
P (ng/g dried feces)
1000
900
800
700
600
500
400
300
200
100
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

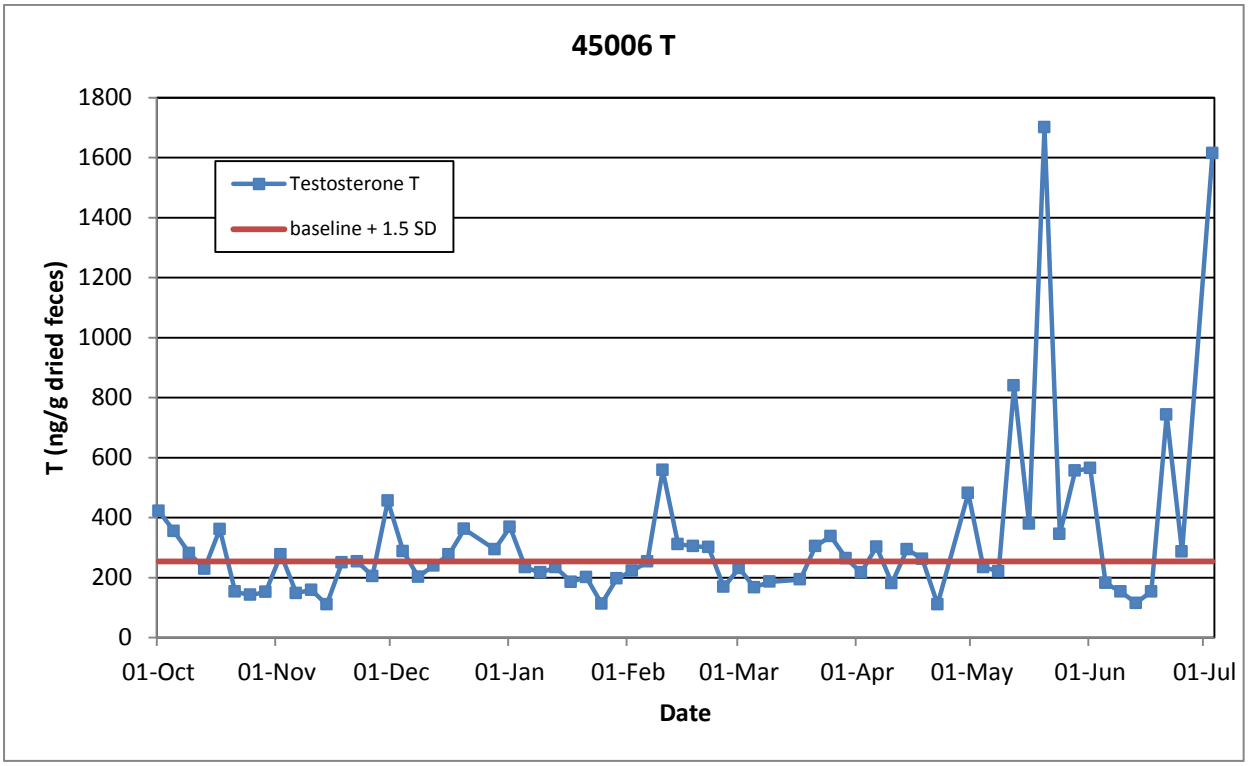

45006
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
1800
1600
1400
1200
1000
800
600
400
200
0
25000
20000
15000
10000
5000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

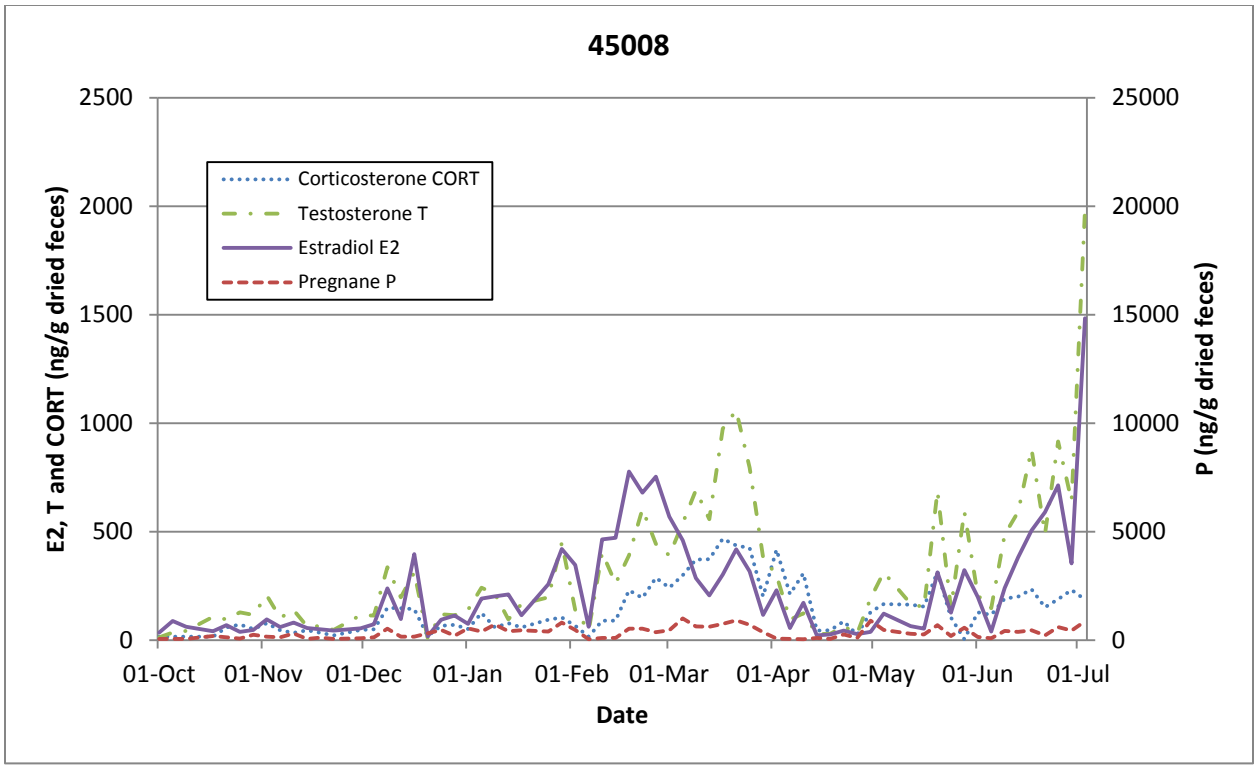

45006 Follicular changes
Length L
Width W
Surface Area SA
L & W (cm) and SA (cm²)
1.2
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

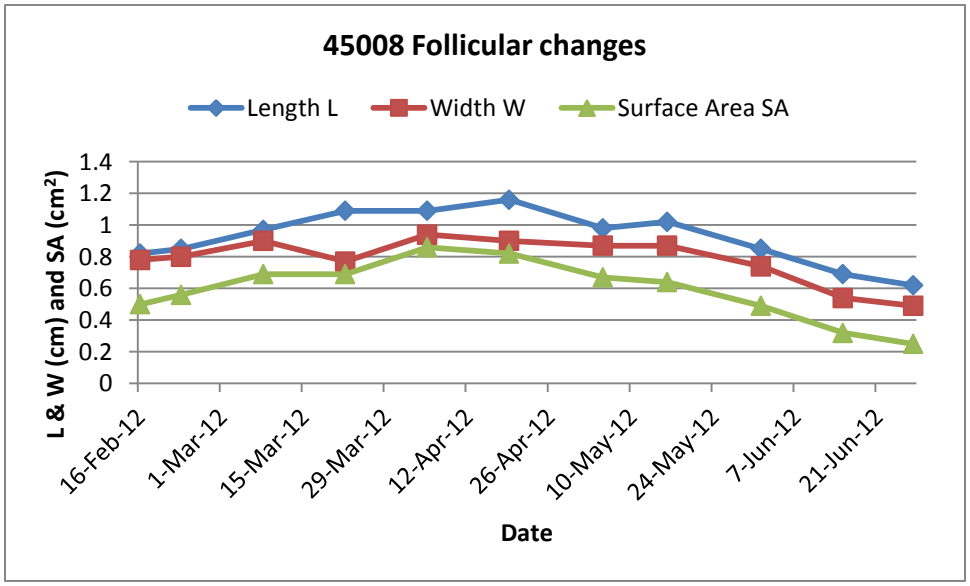

45006 CORT
Corticosterone CORT
baseline + 1.5 SD
CORT (ng/g dried feces)
400
350
300
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

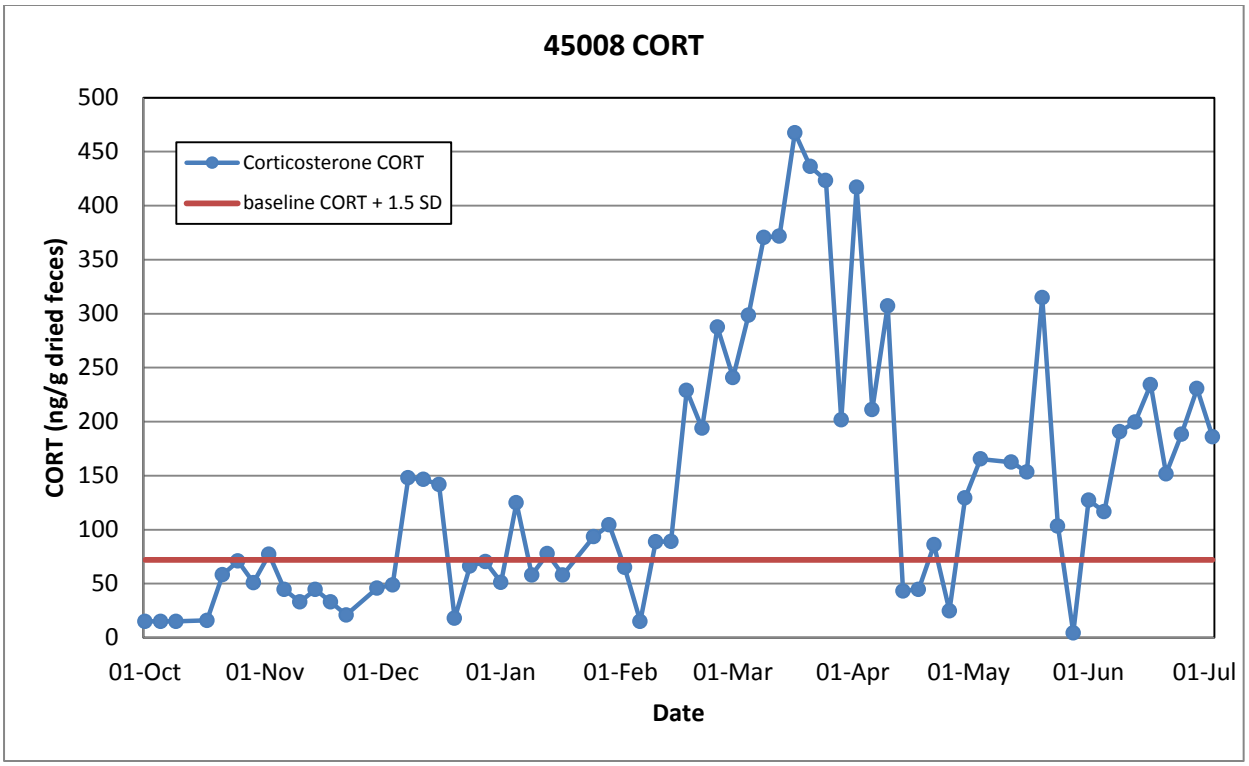

45006 E2
Estradiol E2
baseline + 1.5 SD
E2 (ng/g dired feces)
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

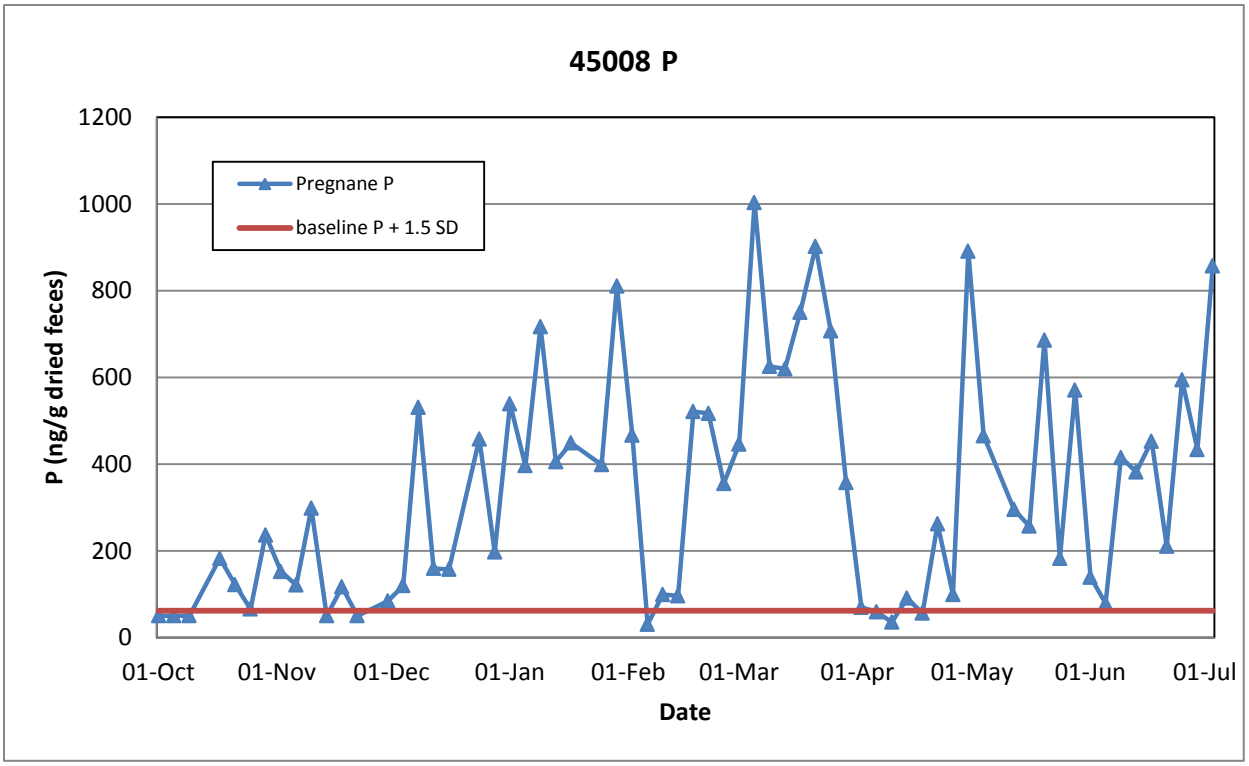

45006 P
3000
2500
2000
1500
1000
500
0
P (ng/g dried feces)
Pregnane P
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

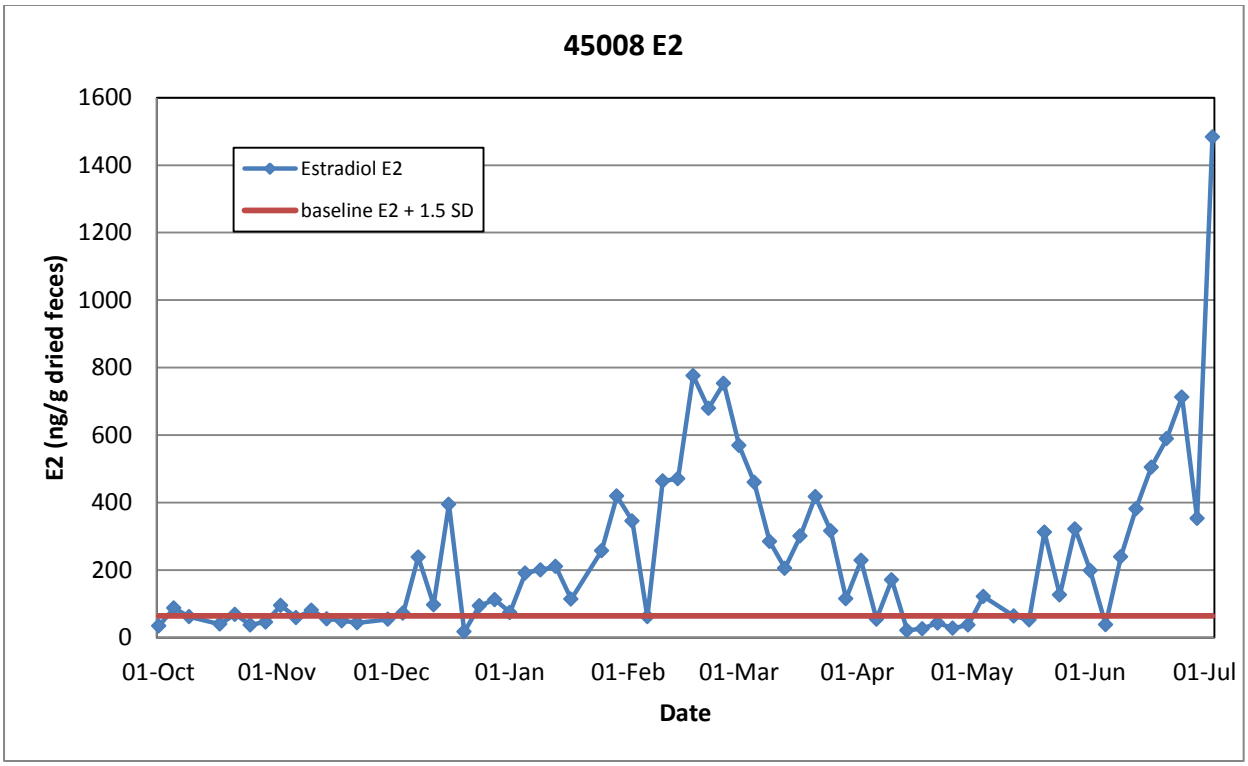

45006 T
1800
1600
1400
1200
1000
800
600
400
200
0
T (ng/g dried feces)
Testosterone T
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

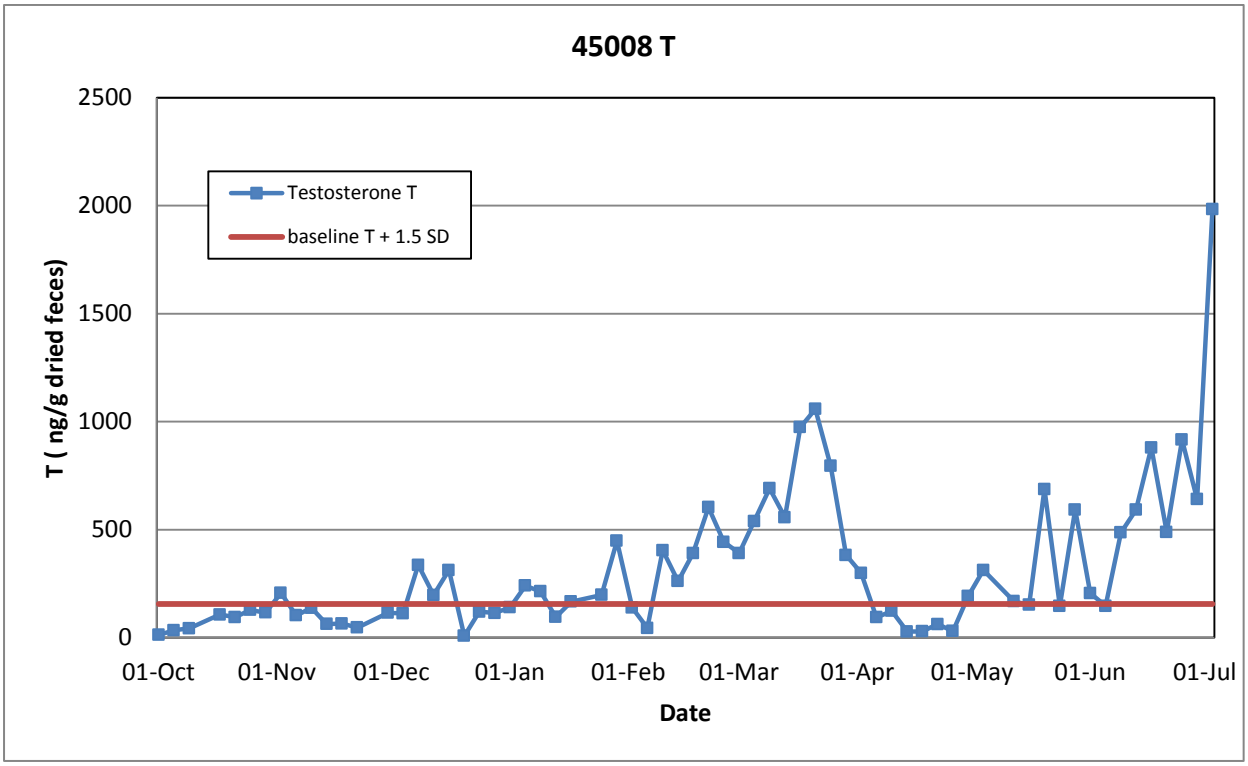

45008
2500
2000
1500
1000
500
0
25000
20000
15000
10000
5000
0
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

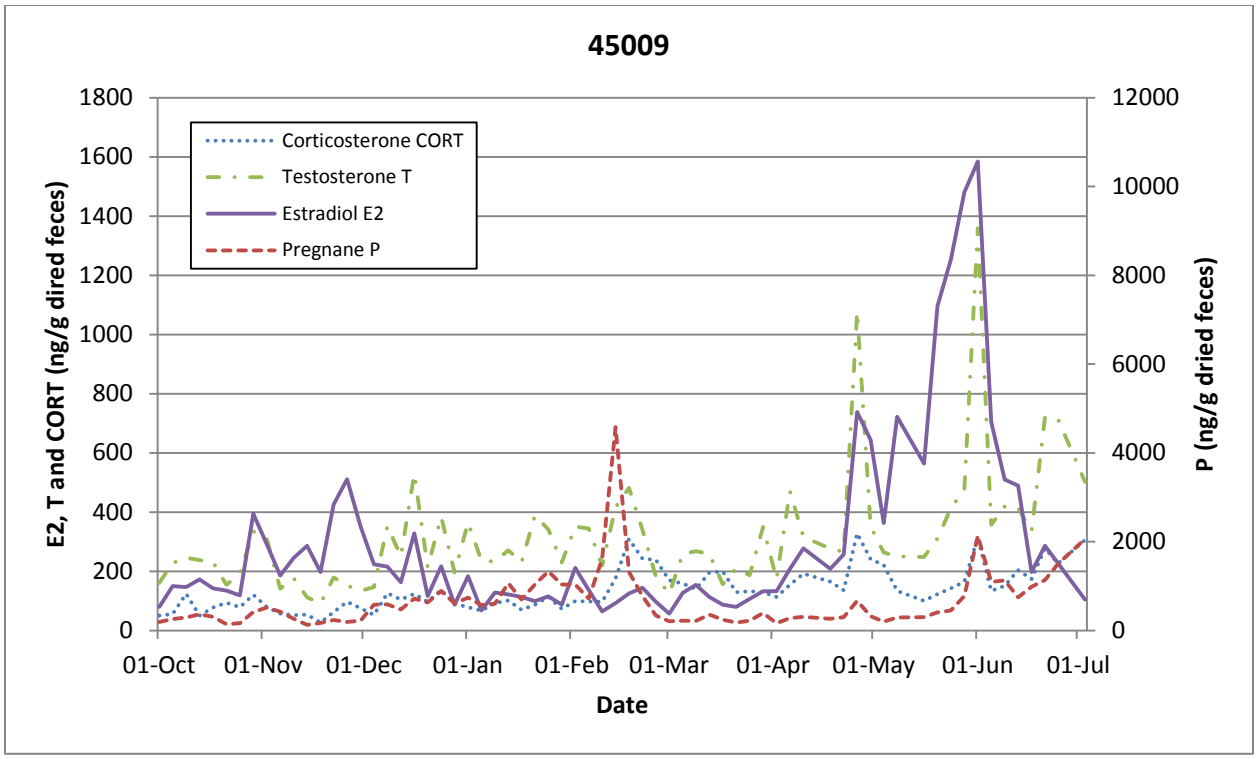

45008 Follicular changes
Length L
Width W
Surface Area SA
1.4
1.2
1
0.8
0.6
0.4
0.2
0
L & W (cm) and SA (cm²)
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

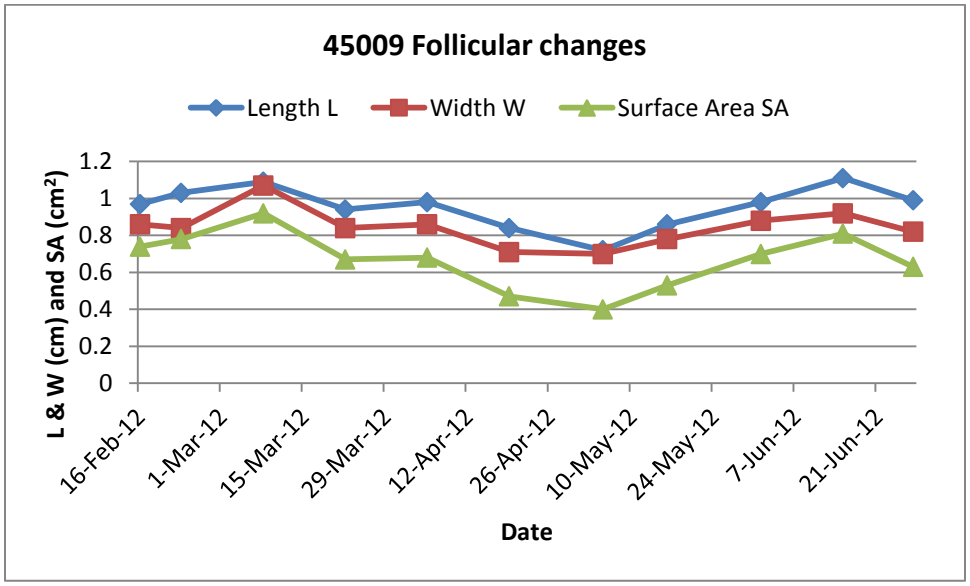

45008 CORT
Corticosterone CORT
baseline CORT + 1.5 SD
CORT (ng/g dried feces)
500
450
400
350
300
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

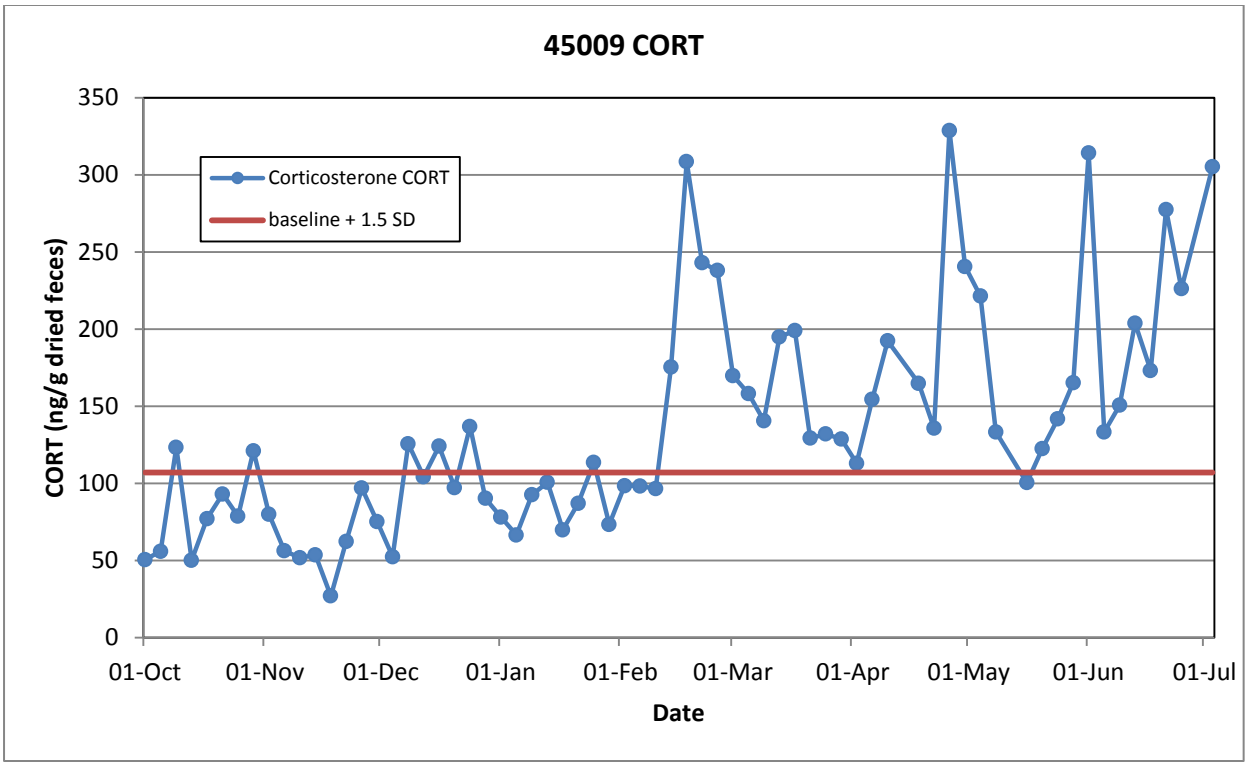

45008 P
Pregnane P
baseline P + 1.5 SD
P (ng/g dried feces)
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

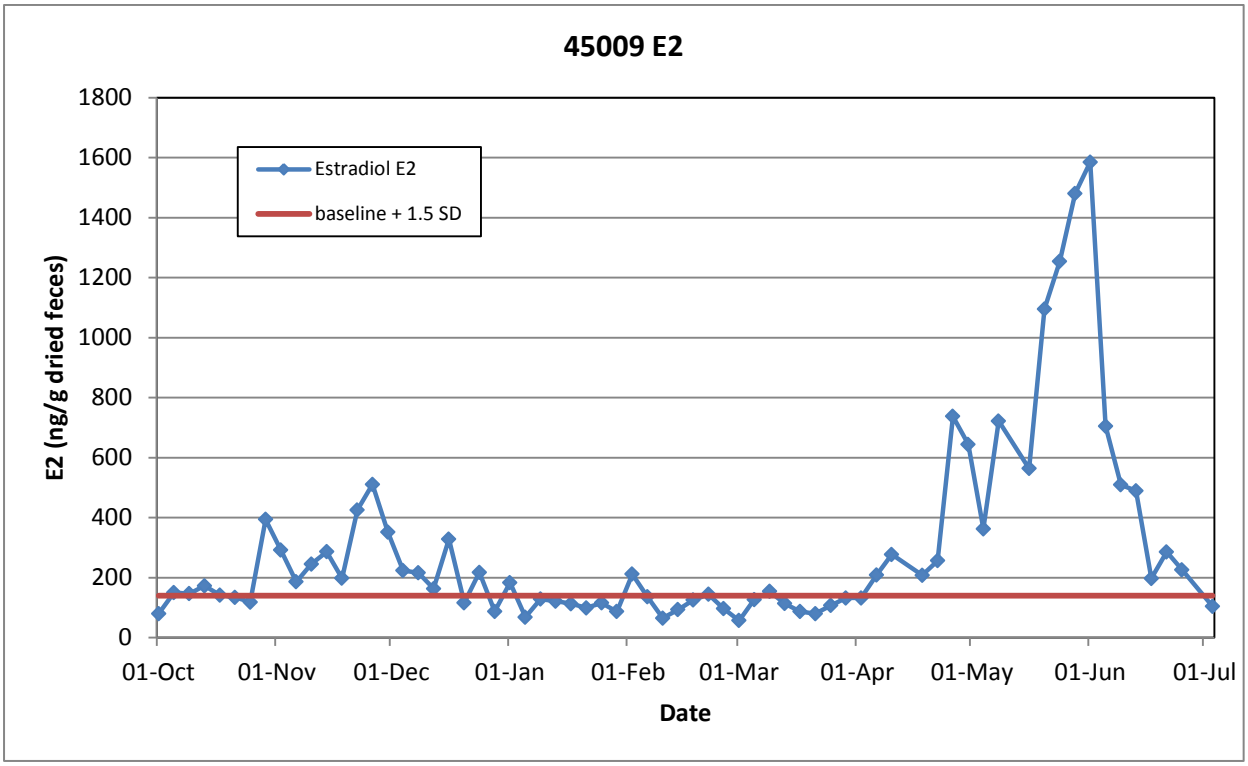

45008 E2
1600
1400
1200
1000
800
600
400
200
0
E2 (ng/g dried feces)
Estradiol E2
baseline E2 + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

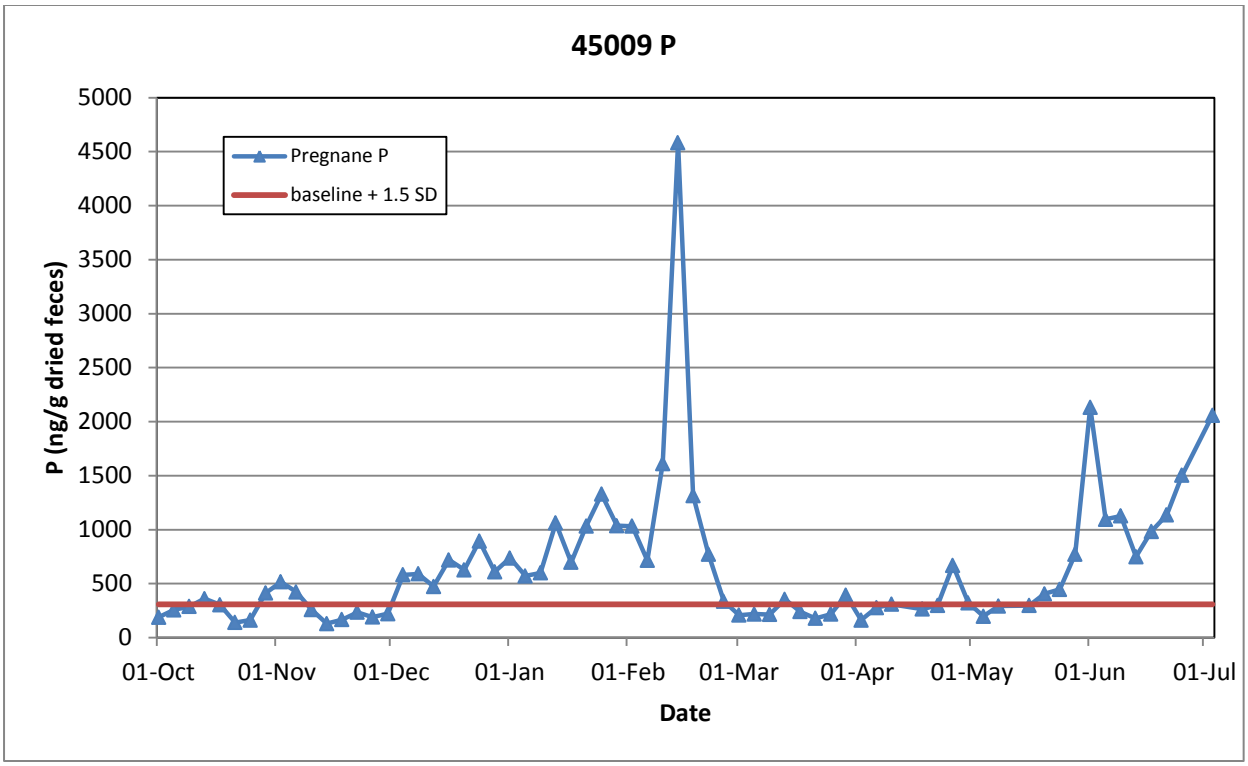

45008 T
2500
2000
1500
1000
500
0
T (ng/g dried feces)
Testosterone T
baseline T + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

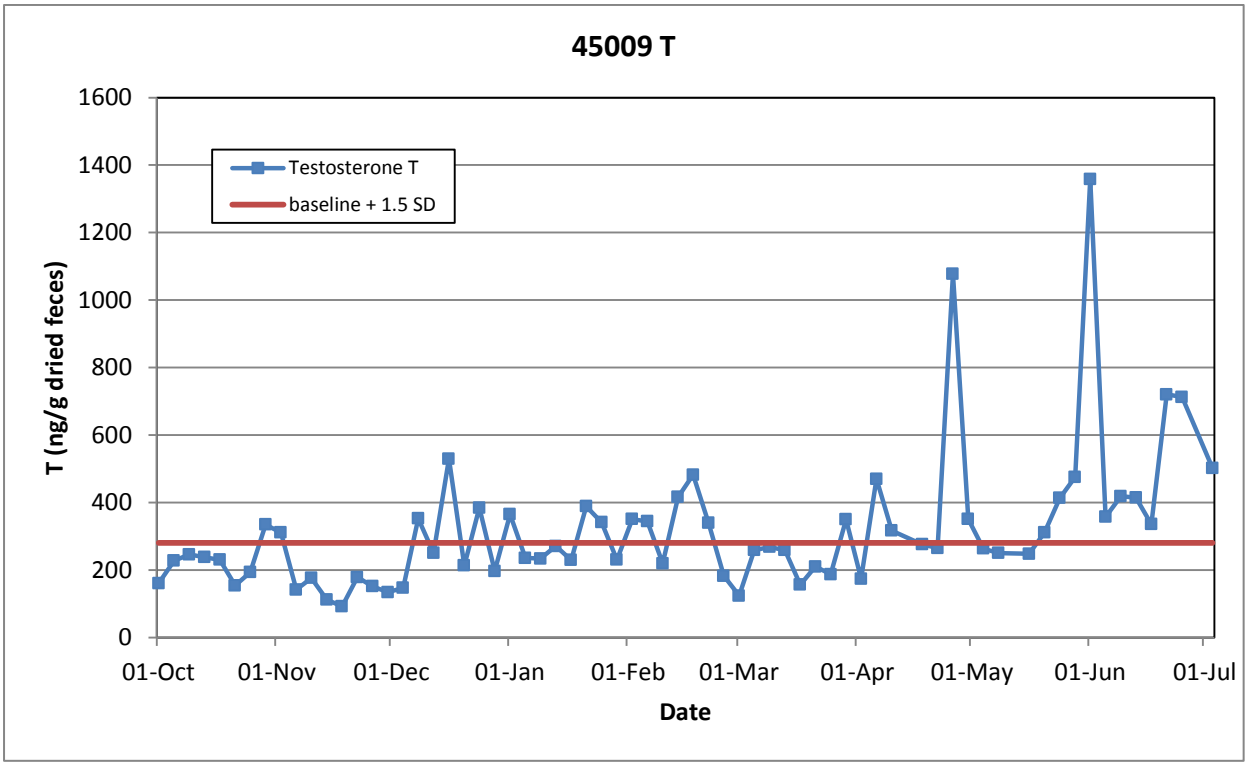

45009
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dired feces)
P (ng/g dried feces)
1800
1600
1400
1200
1000
800
600
400
200
0
12000
10000
8000
6000
4000
2000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

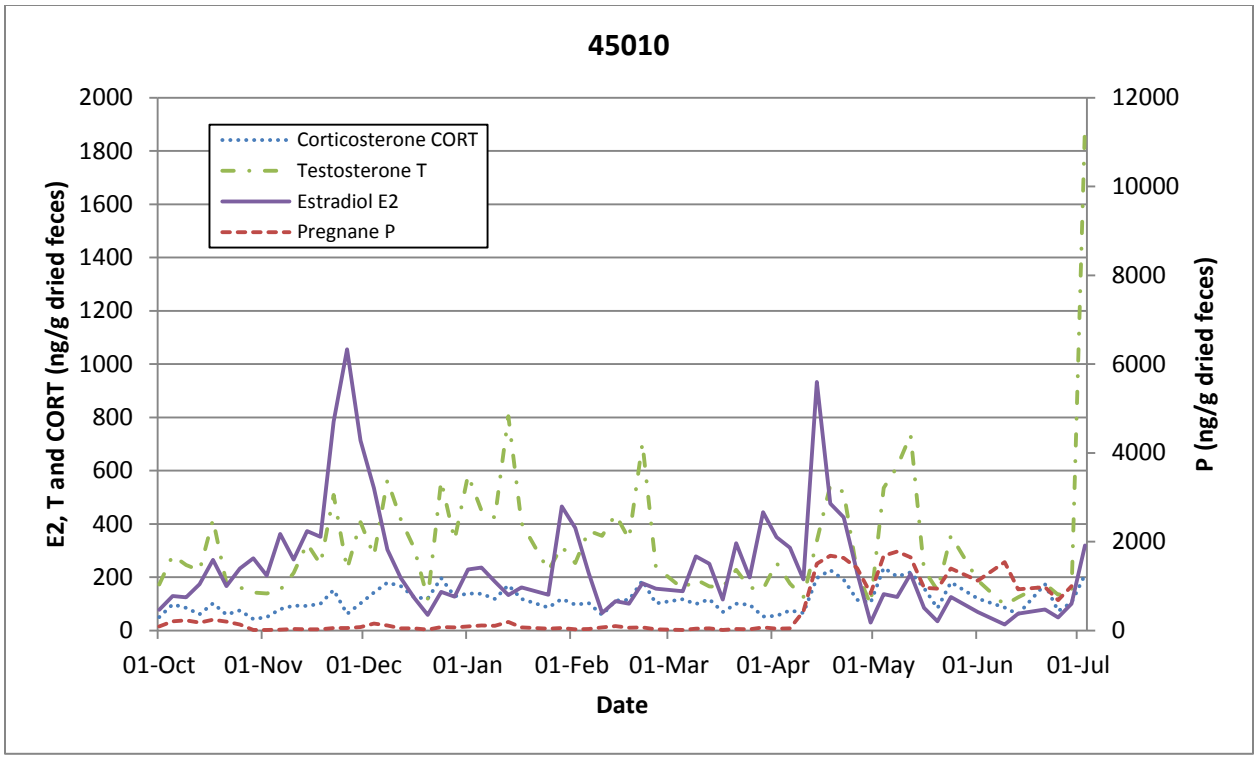

45009 Follicular changes
Length L
Width W
Surface Area SA
L & W (cm) and SA (cm²)
1.2
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

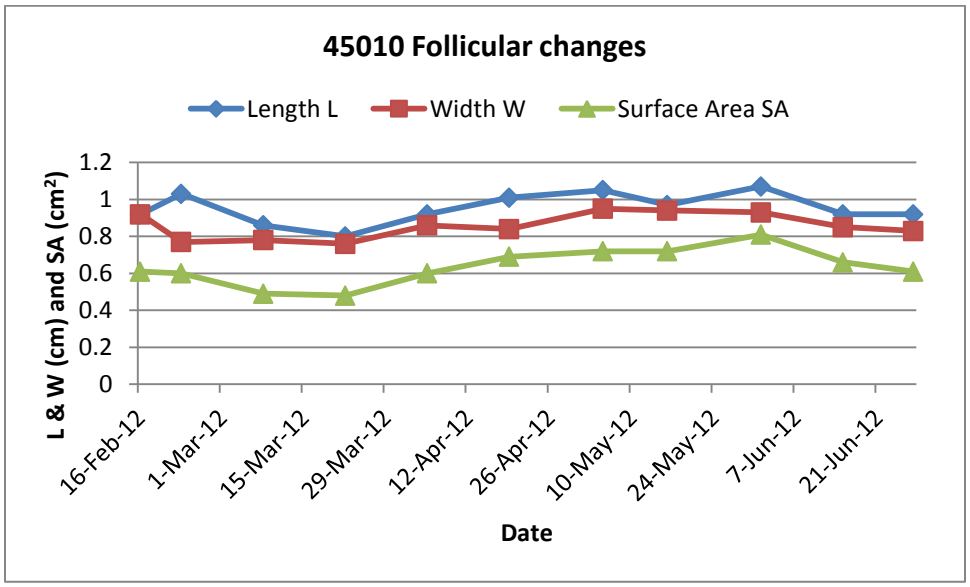

45009 CORT
Corticosterone CORT
baseline + 1.5 SD
CORT (ng/g dried feces)
350
300
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

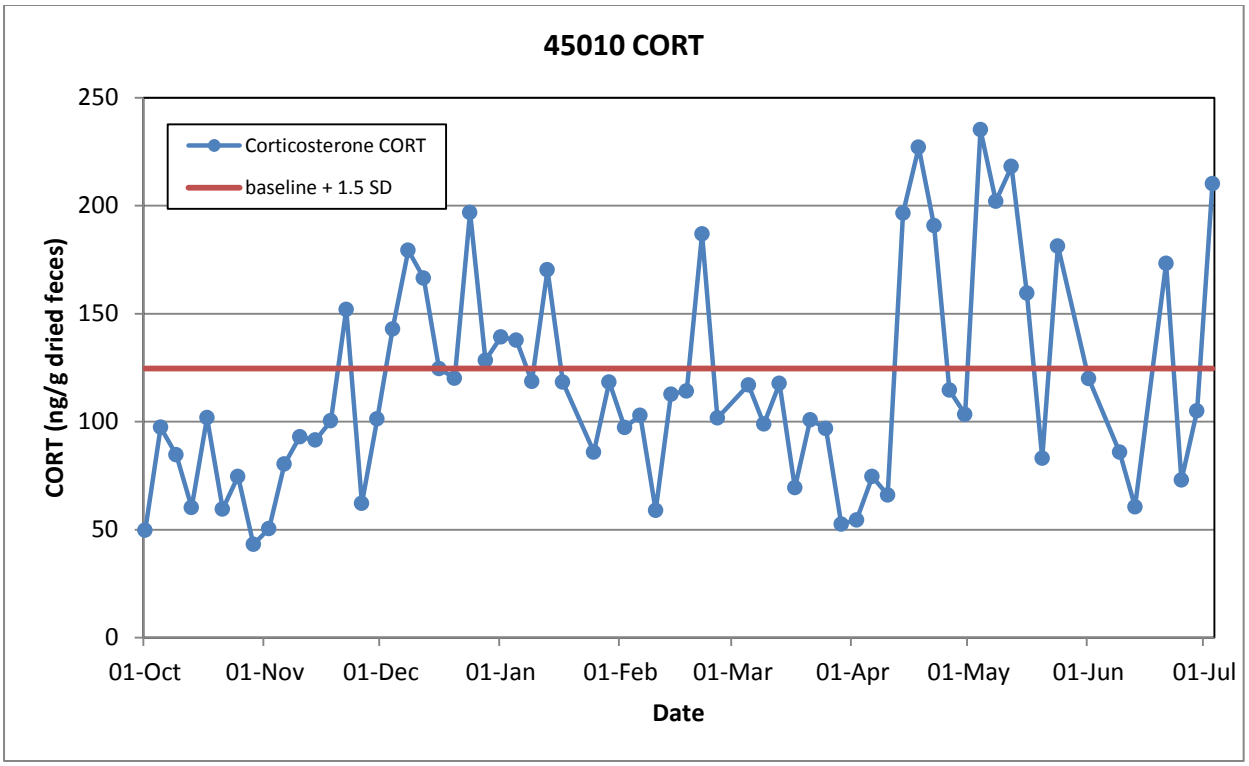

45009 E2
Estradiol E2
baseline + 1.5 SD
E2 (ng/g dried feces)
1800
1600
1400
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

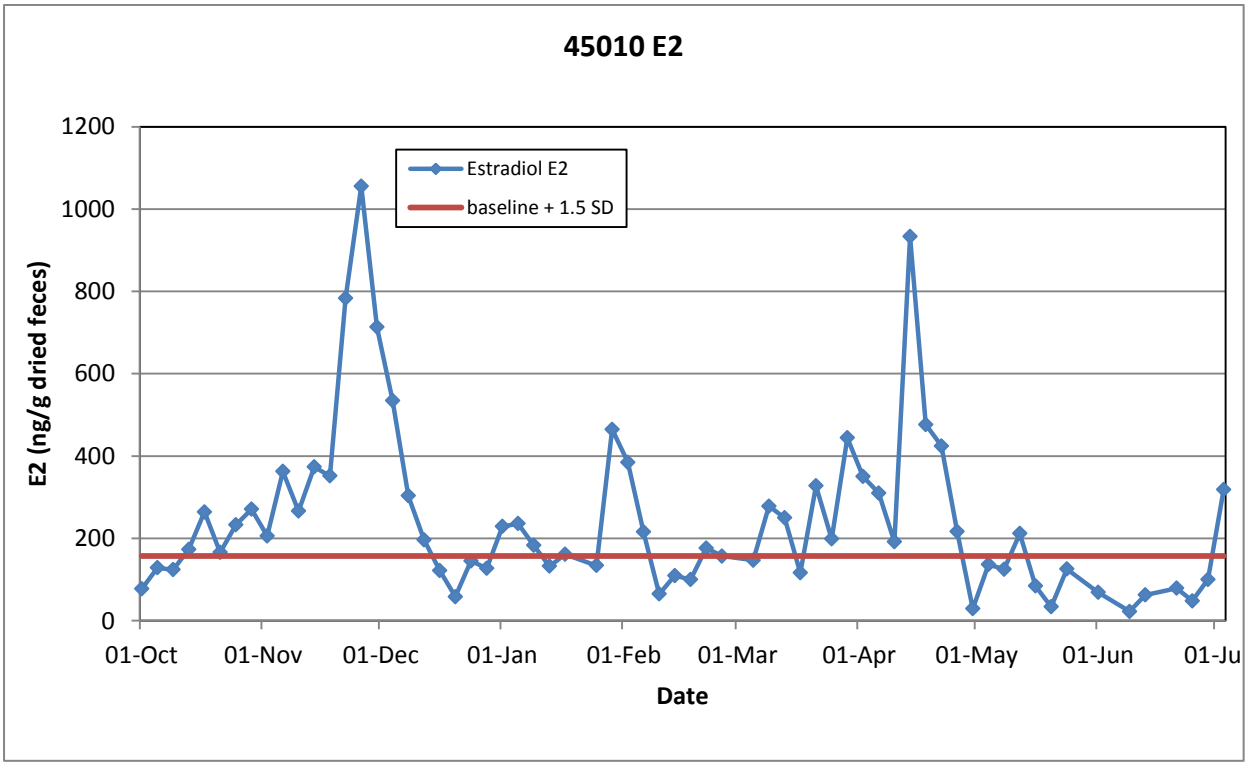

45009 P
Pregnane P
baseline + 1.5 SD
P (ng/g dried feces)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

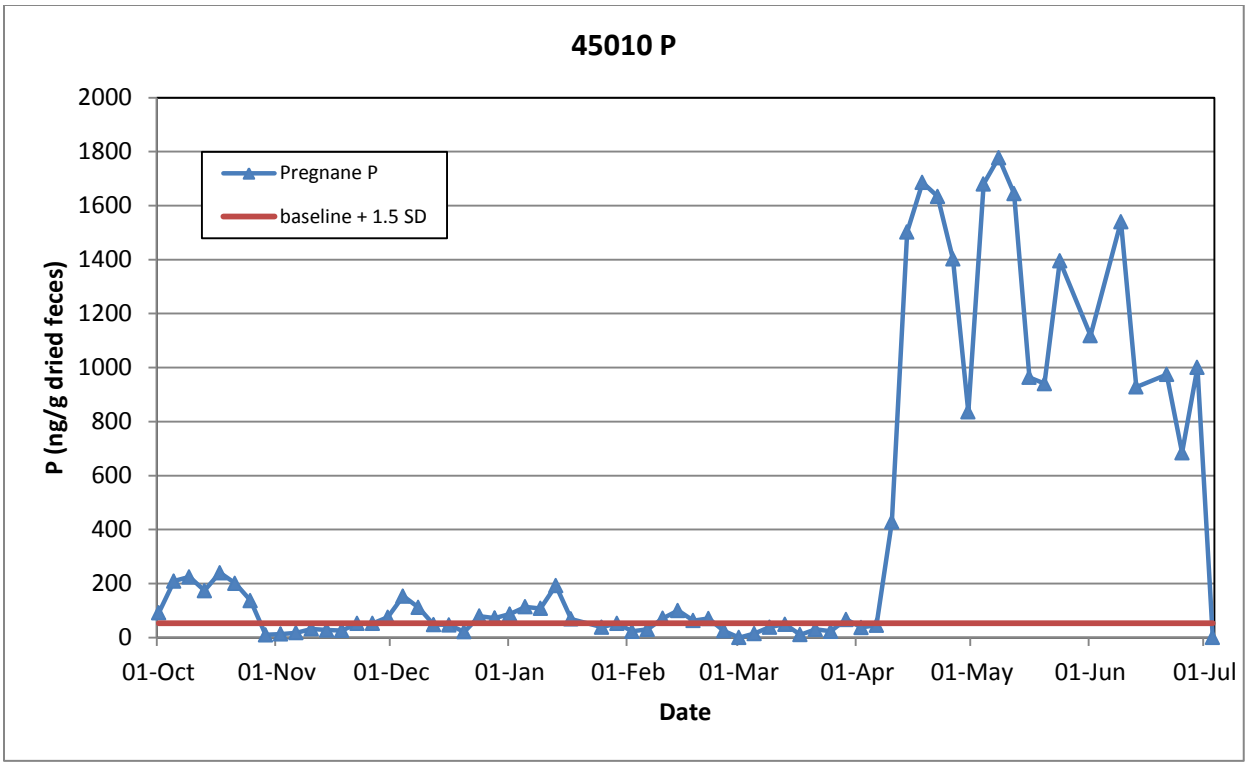

45009 T
Testosterone T
baseline + 1.5 SD
T (ng/g dried feces)
1600
1400
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

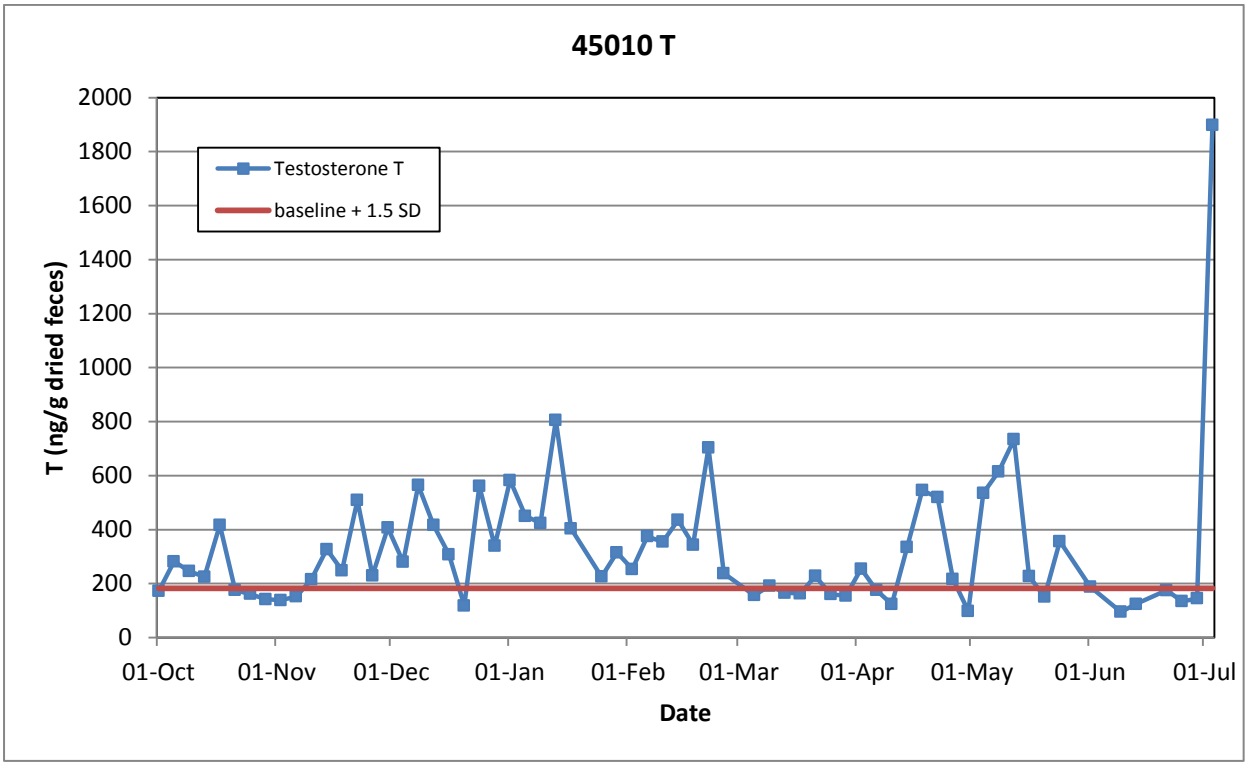

45010
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
12000
10000
8000
6000
4000
2000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

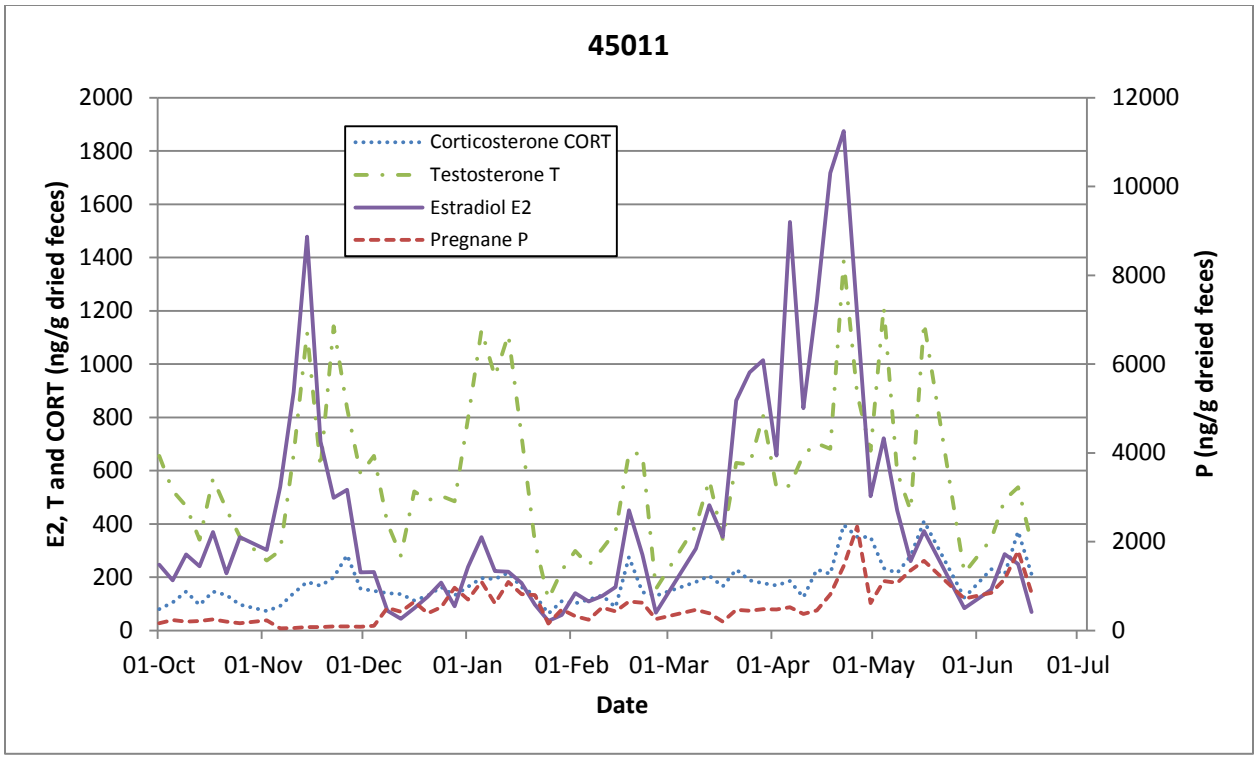

45010 Follicular changes
Length L
Width W
Surface Area SA
L & W (cm) and SA (cm²)
1.2
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

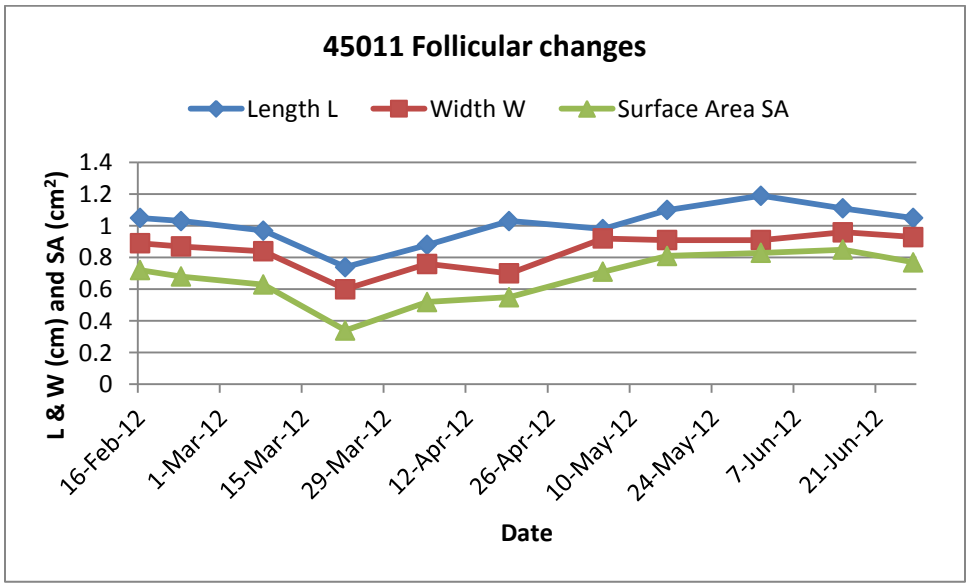

45010 CORT
Corticosterone CORT
baseline + 1.5 SD
CORT (ng/g dried feces)
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

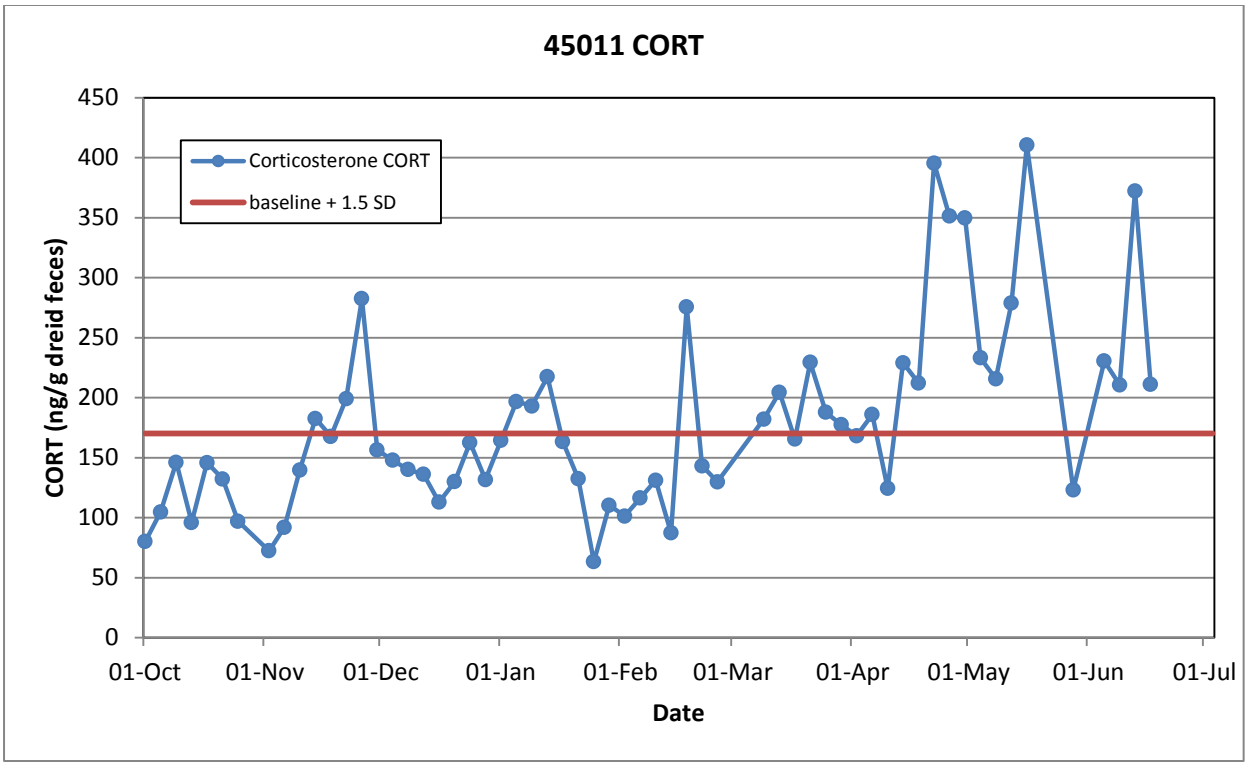

45010 E2
Estradiol E2
baseline + 1.5 SD
E2 (ng/g dried feces)
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Ju
Date

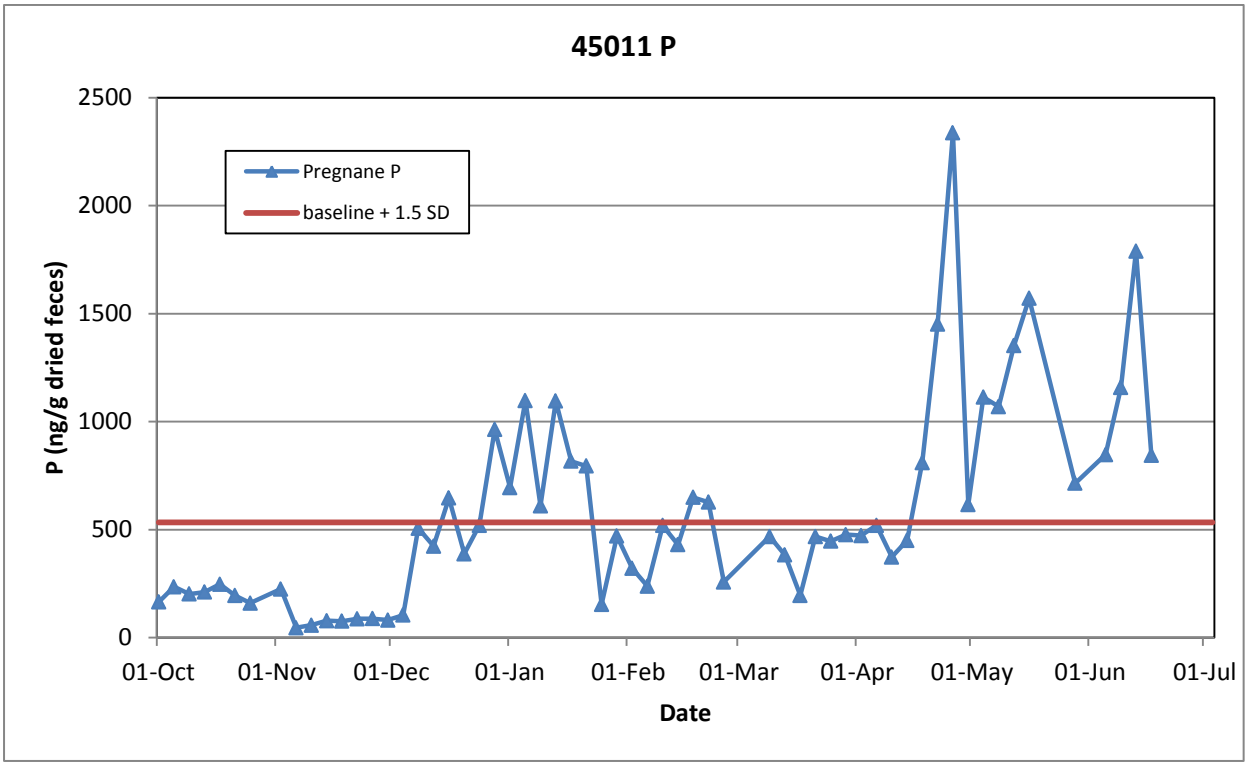

45010 P
Pregnane P
baseline + 1.5 SD
P (ng/g dried feces)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

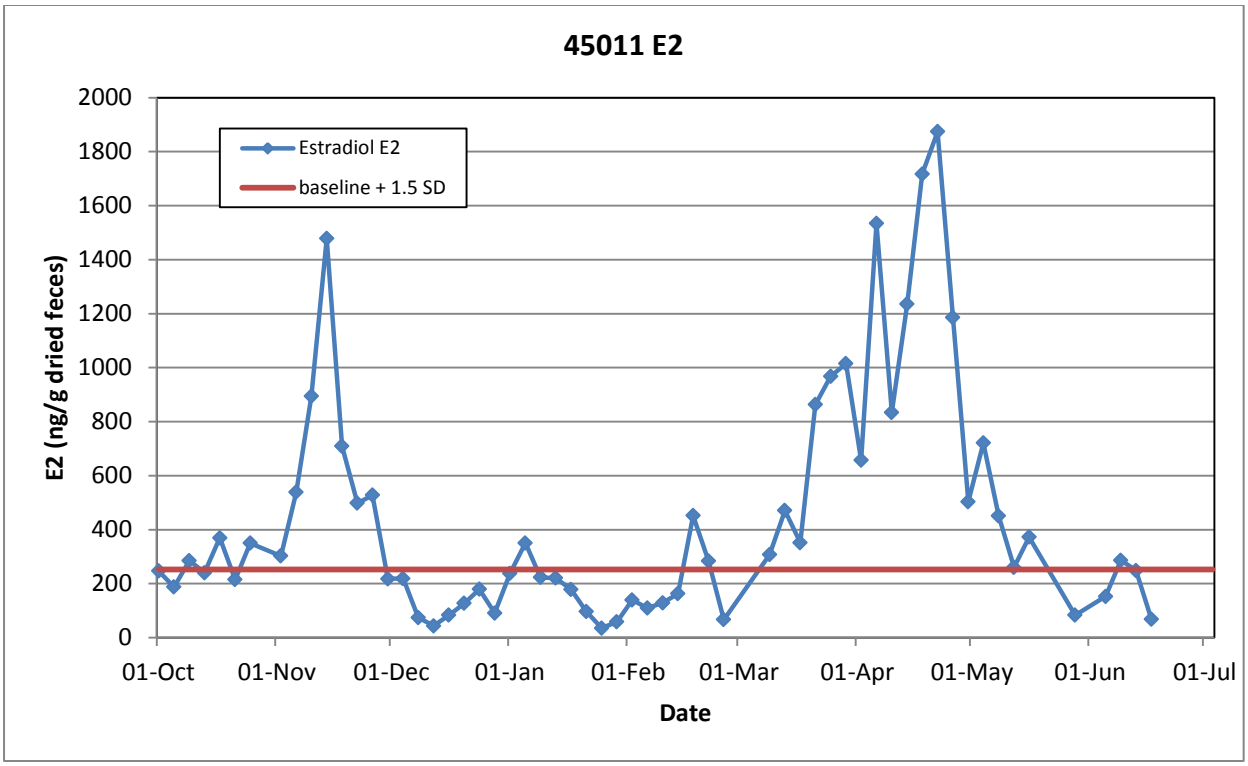

45010 T
Testosterone T
baseline + 1.5 SD
T (ng/g dried feces)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

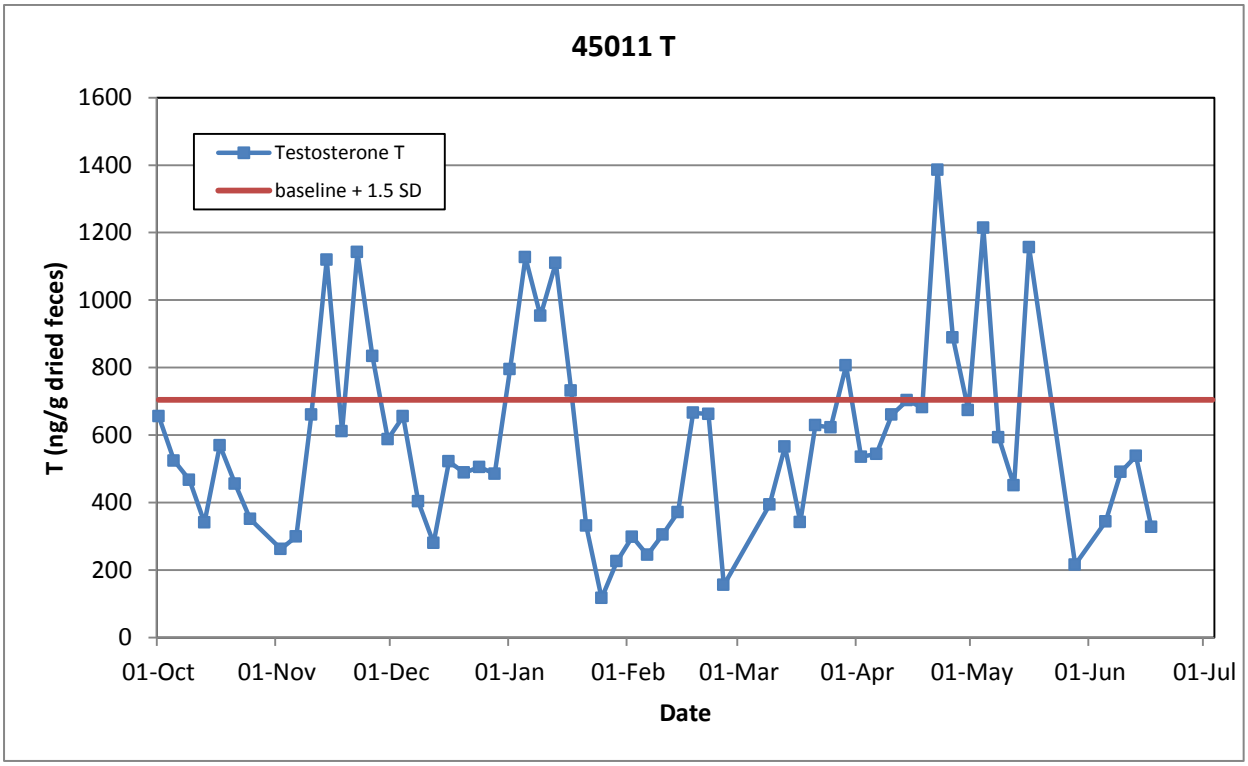

45011
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dreied feces)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
12000
10000
8000
6000
4000
2000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

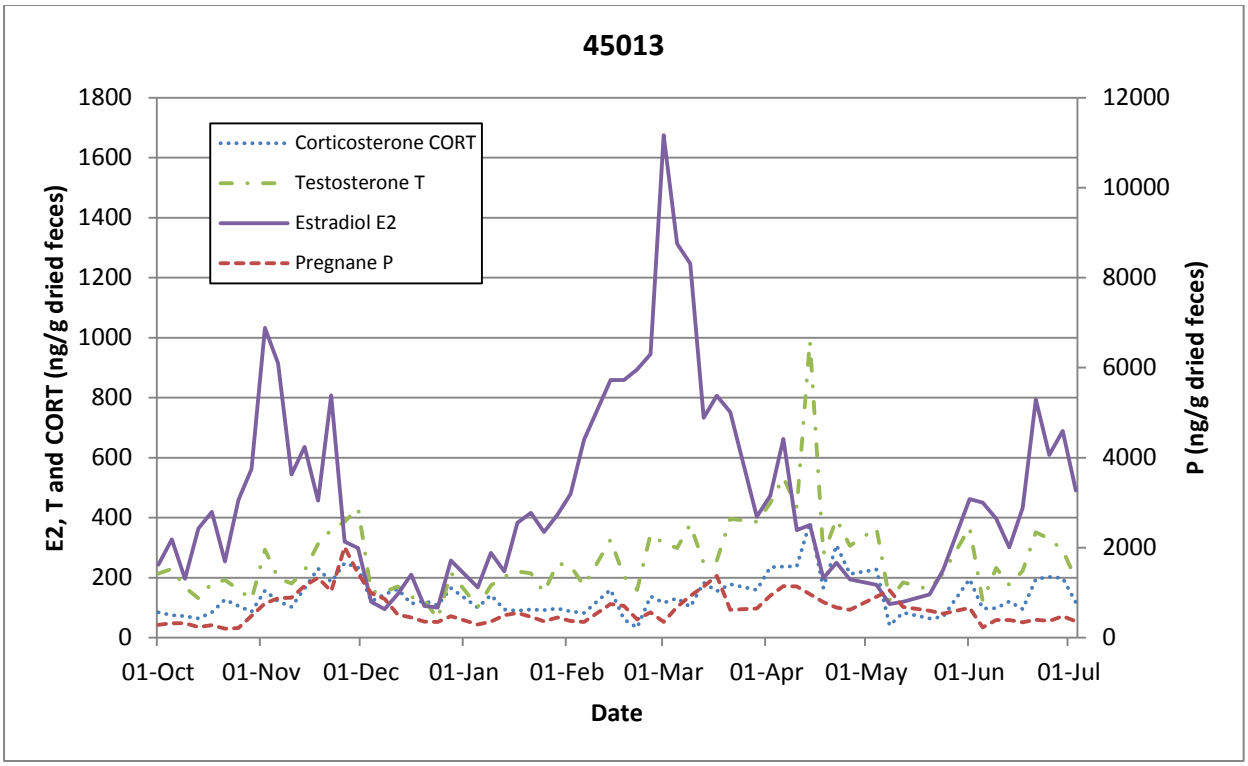

45011 Follicular changes
Length L
Width W
Surface Area SA
L & W (cm) and SA (cm²)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

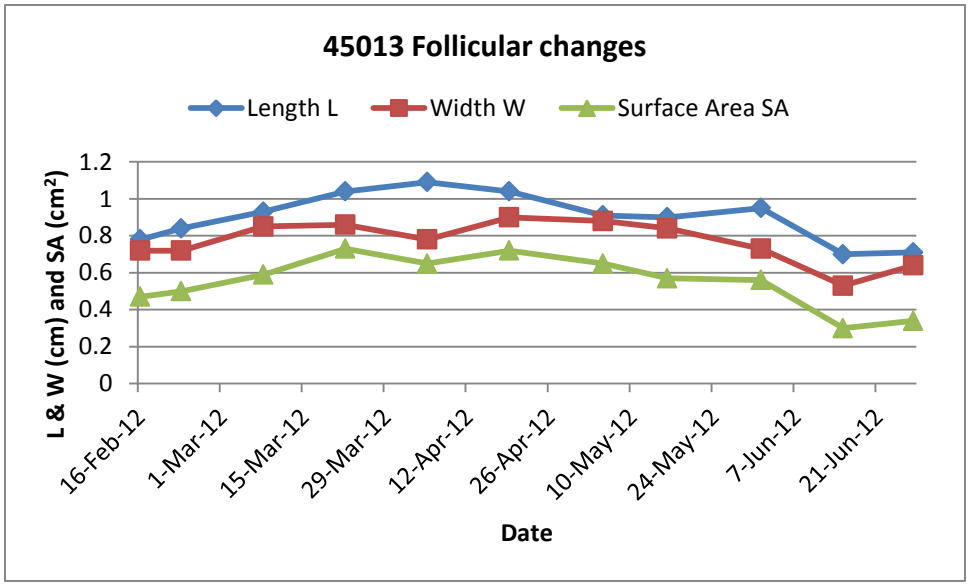

45011 CORT
Corticosterone CORT
baseline + 1.5 SD
CORT (ng/g dreid feces)
450
400
350
300
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

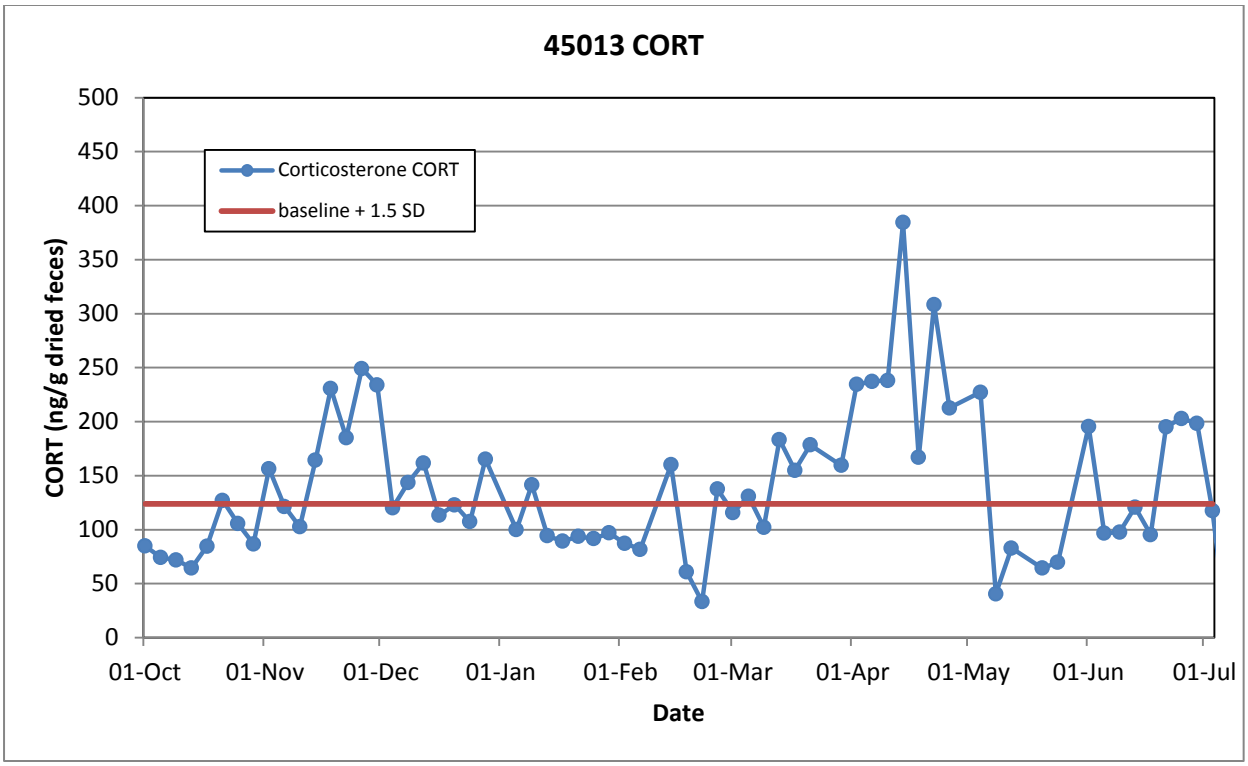

45011 P
Pregnane P
baseline + 1.5 SD
P (ng/g dried feces)
2500
2000
1500
1000
500
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

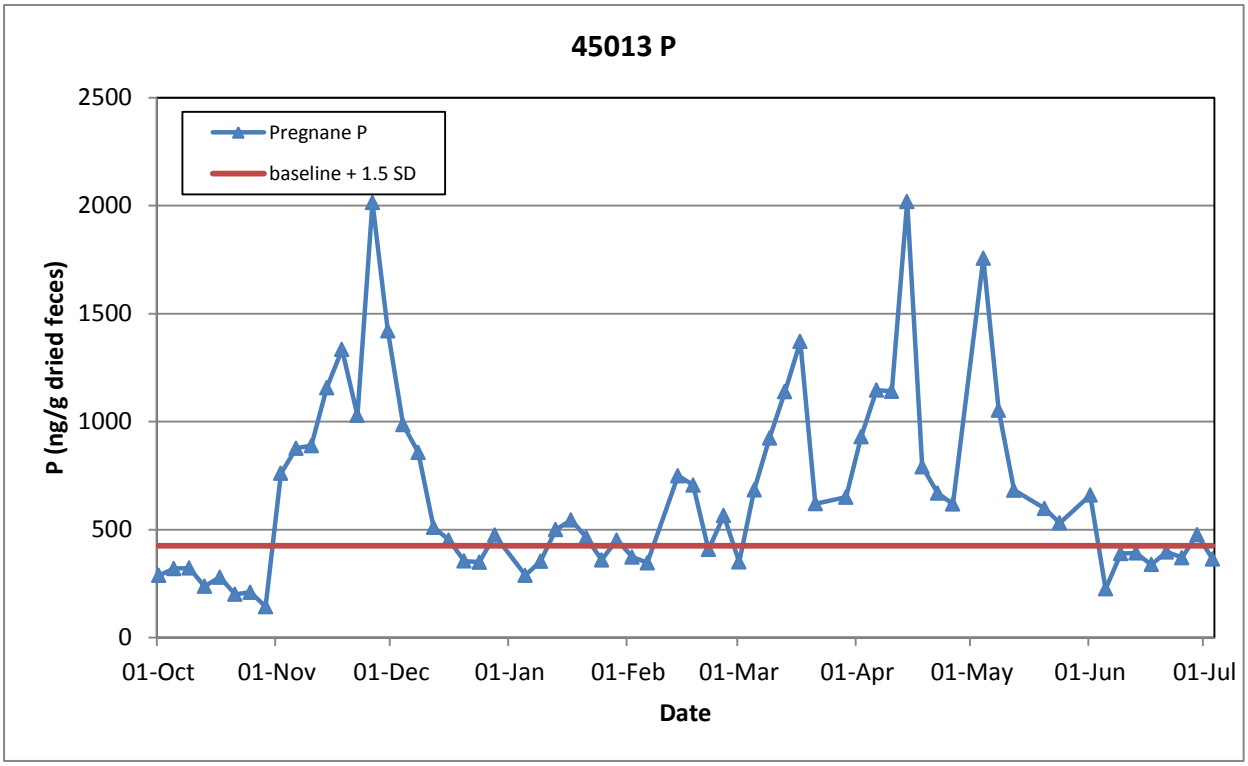

45011 E2
Estradiol E2
baseline + 1.5 SD
E2 (ng/g dried feces)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

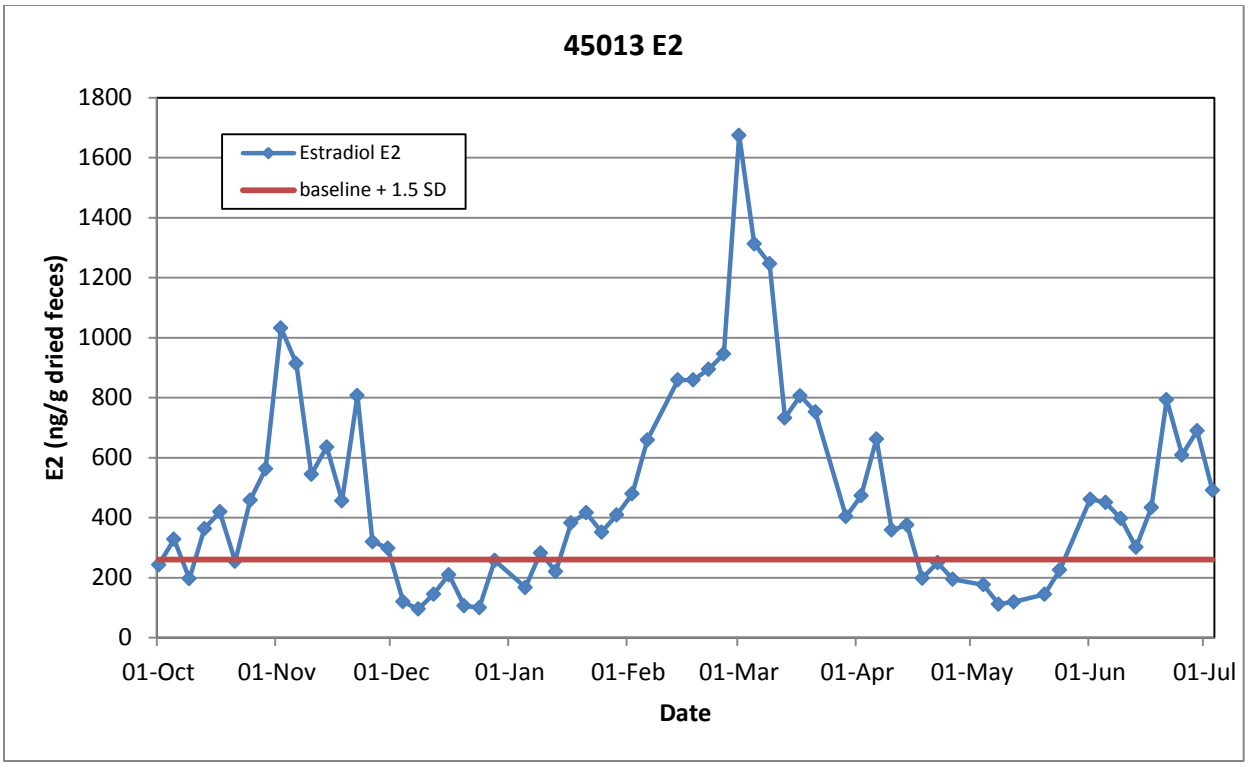

45011 T
Testosterone T
baseline + 1.5 SD
T (ng/g dried feces)
1600
1400
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

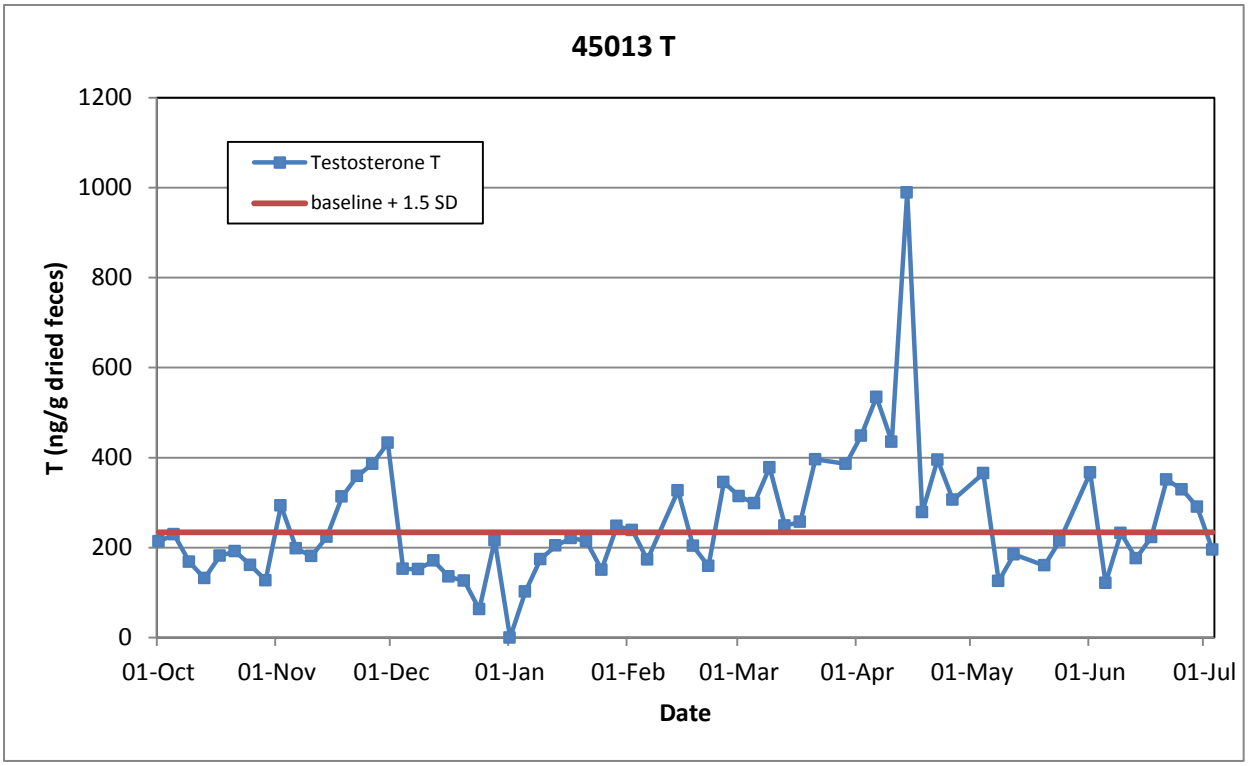

45013
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
1800
1600
1400
1200
1000
800
600
400
200
0
12000
10000
8000
6000
4000
2000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

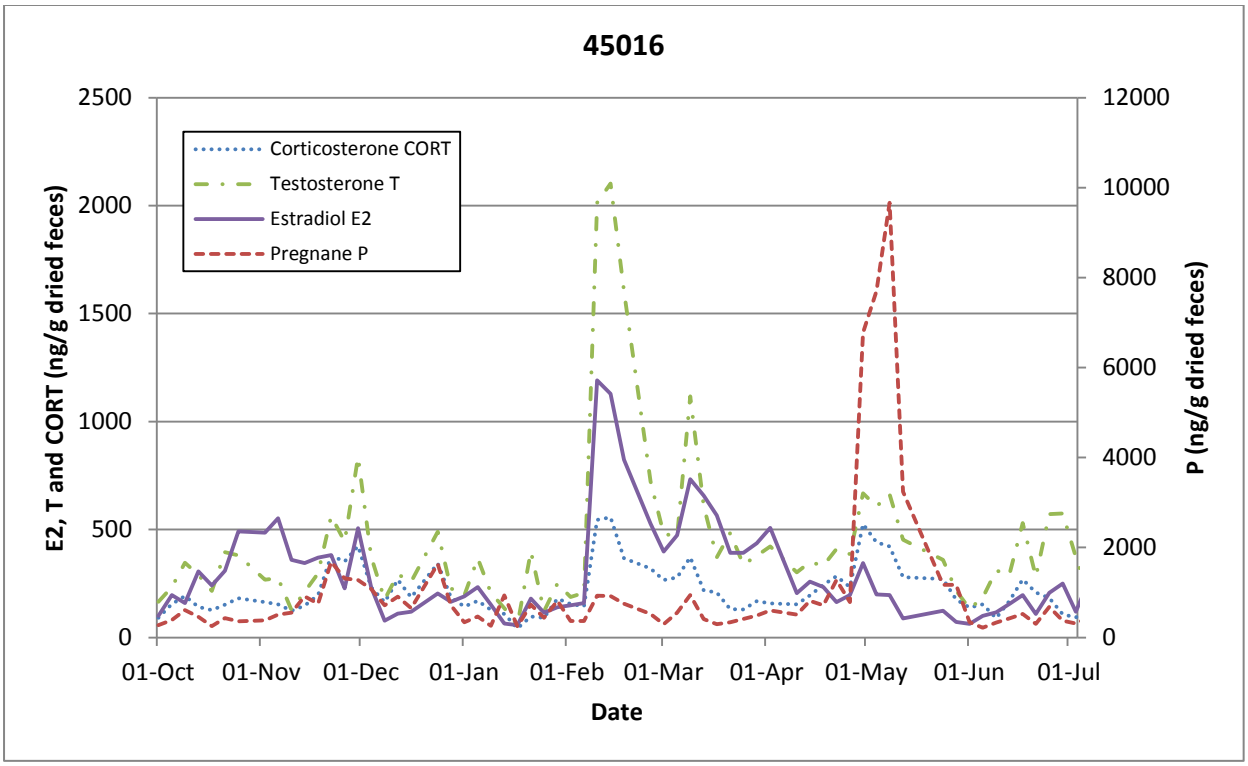

45013 Follicular changes
Length L
Width W
Surface Area SA
L & W (cm) and SA (cm²)
1.2
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

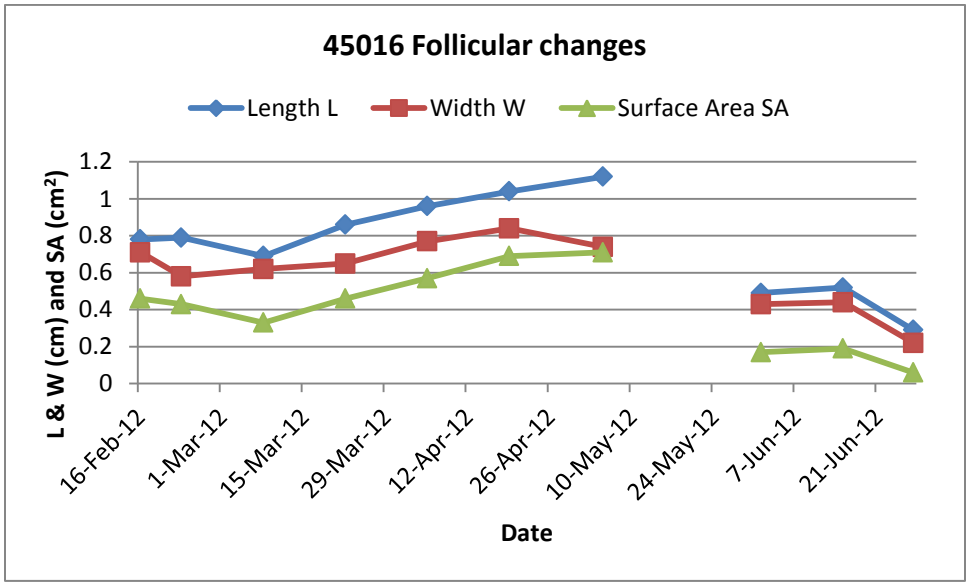

45013 CORT
Corticosterone CORT
baseline + 1.5 SD
CORT (ng/g dried feces)
500
450
400
350
300
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

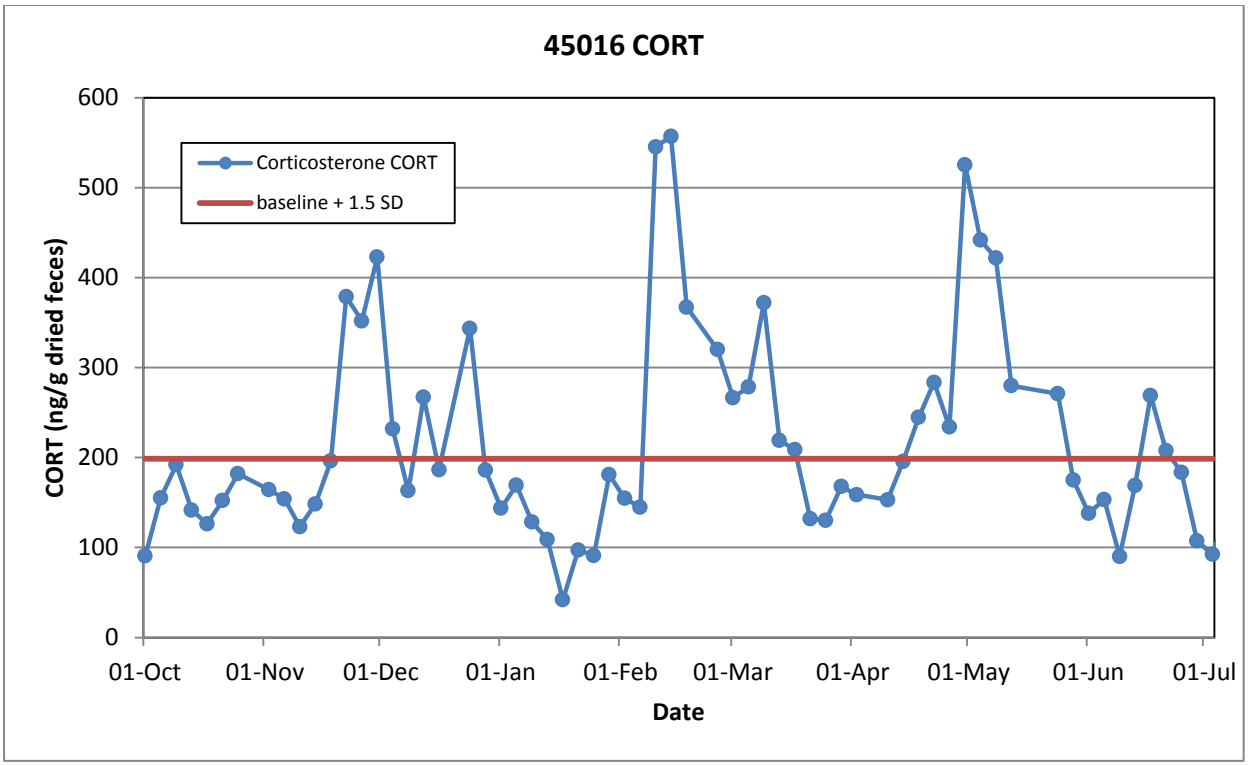

45013 P
Pregnane P
baseline + 1.5 SD
P (ng/g dried feces)
2500
2000
1500
1000
500
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

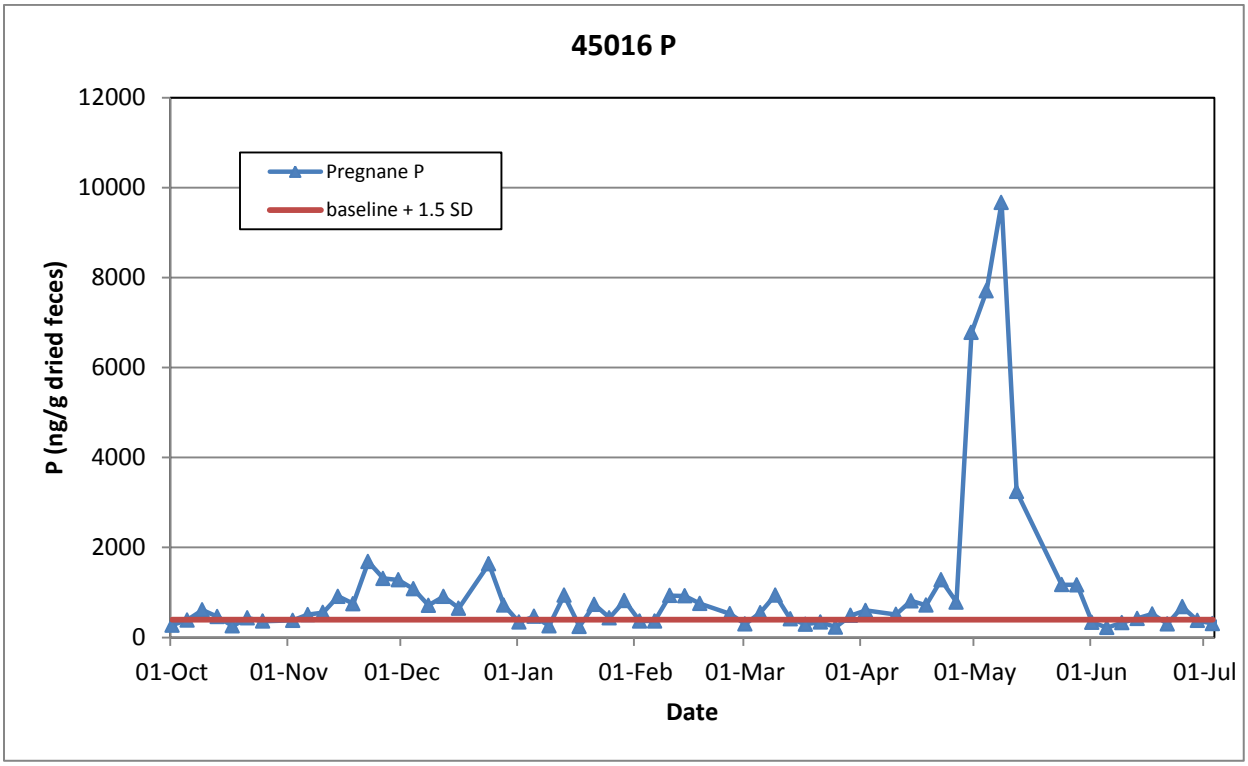

45013 E2
Estradiol E2
baseline + 1.5 SD
E2 (ng/g dried feces)
1800
1600
1400
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

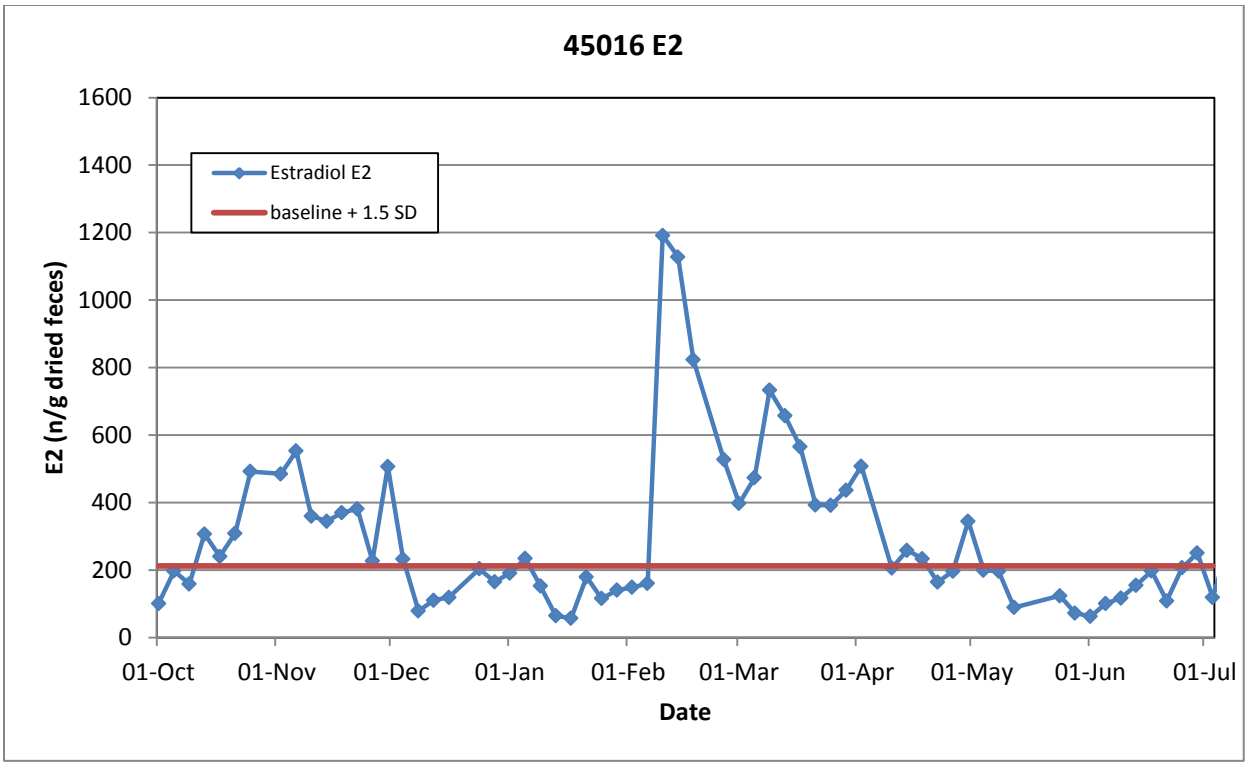

45013 T
Testosterone T
baseline + 1.5 SD
T (ng/g dried feces)
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

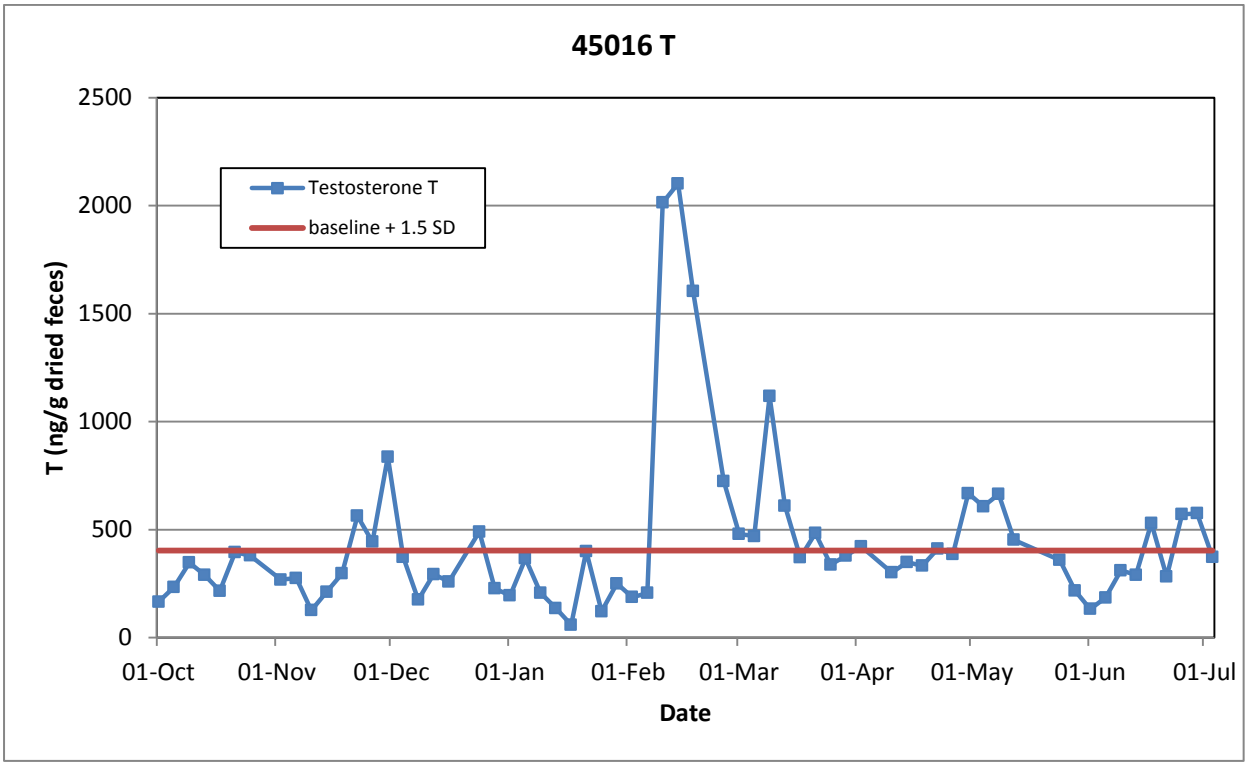

45016
2500
2000
1500
1000
500
0
12000
10000
8000
6000
4000
2000
0
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

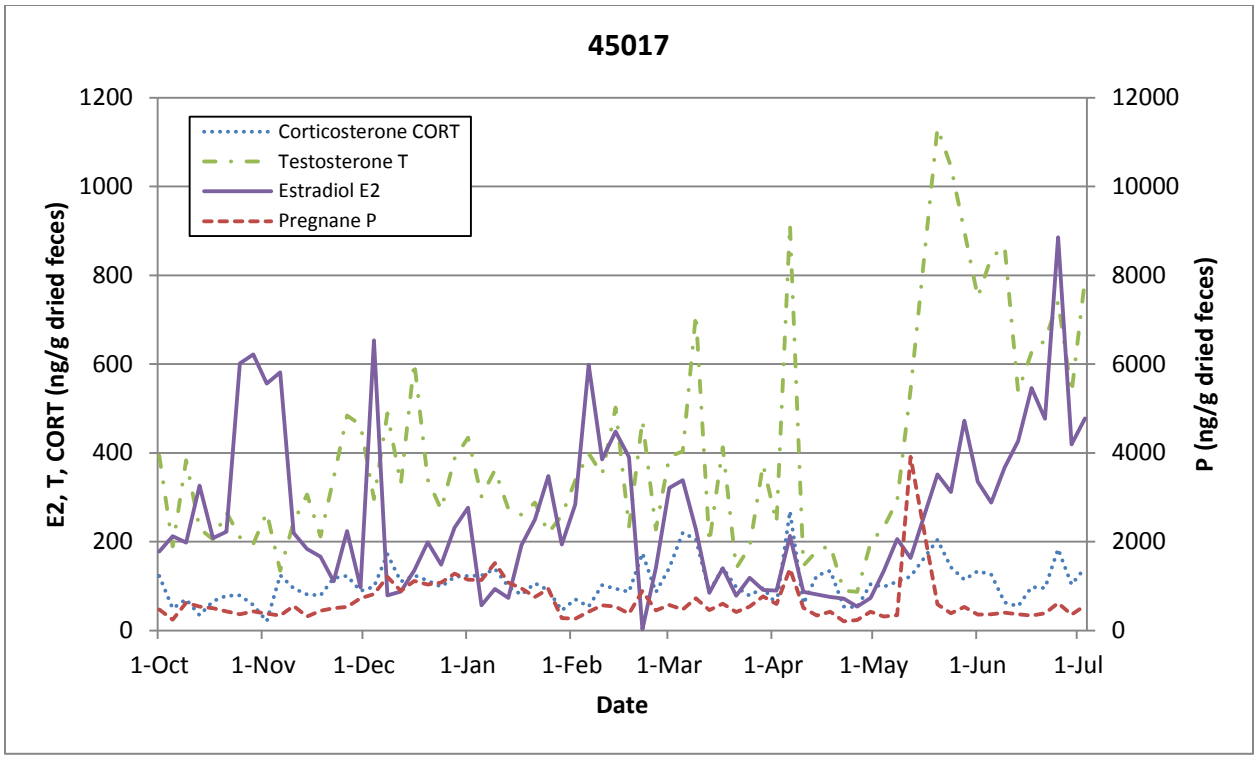

45016 Follicular changes
Length L
Width W
Surface Area SA
1.2
1
0.8
0.6
0.4
0.2
0
L & W (cm) and SA (cm²)
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

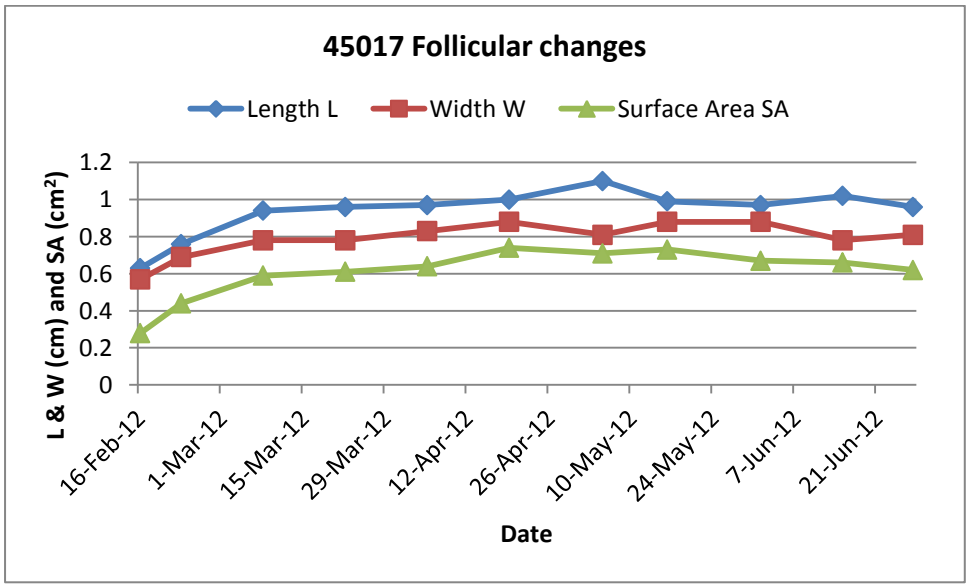

45016 CORT
Corticosterone CORT
baseline + 1.5 SD
CORT (ng/g dried feces)
600
500
400
300
200
100
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

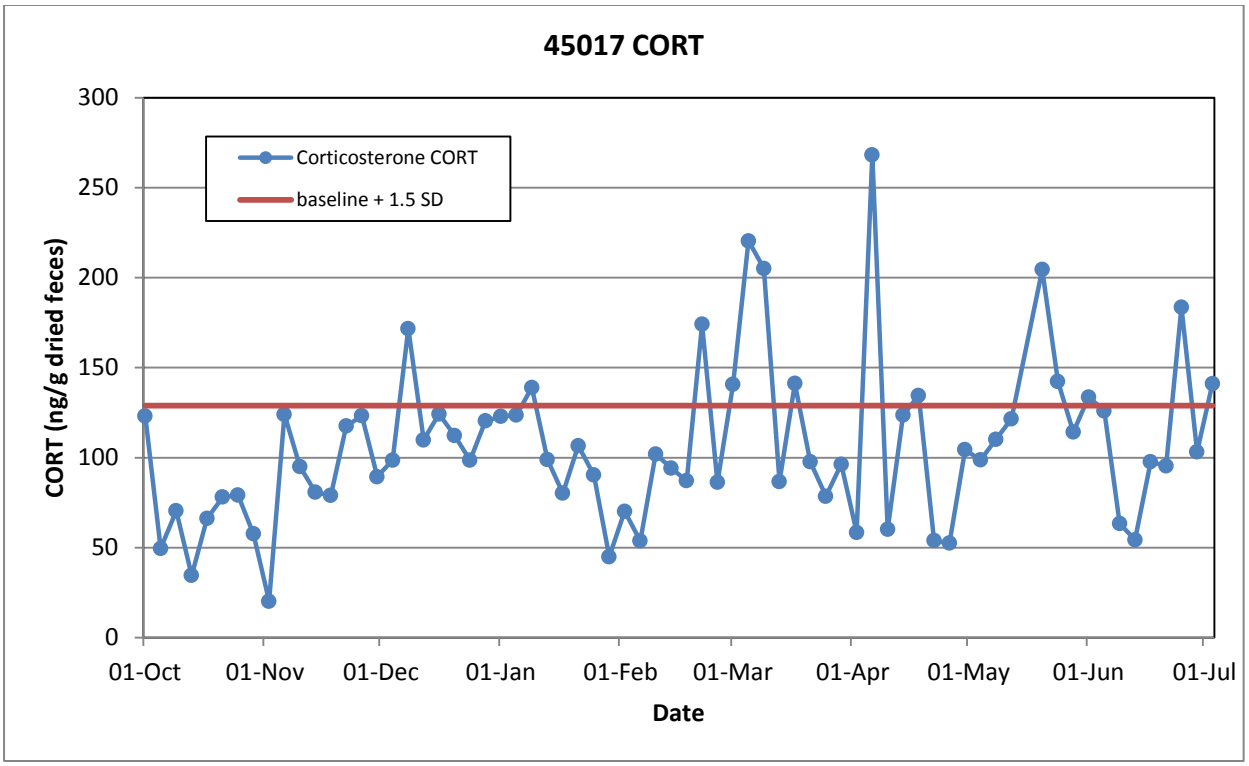

45016 P
Pregnane P
baseline + 1.5 SD
P (ng/g dried feces)
12000
10000
8000
6000
4000
2000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

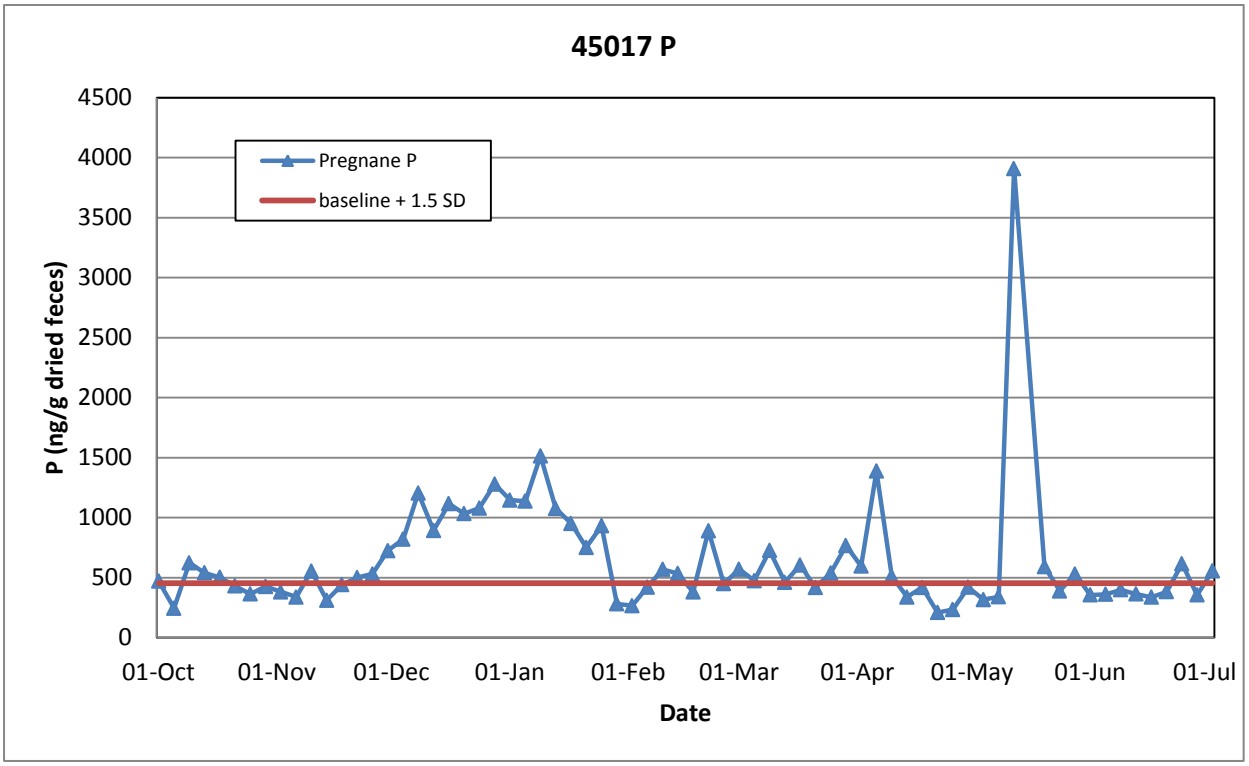

45016 E2
1600
1400
1200
1000
800
600
400
200
0
E2 (n/g dried feces)
Estradiol E2
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

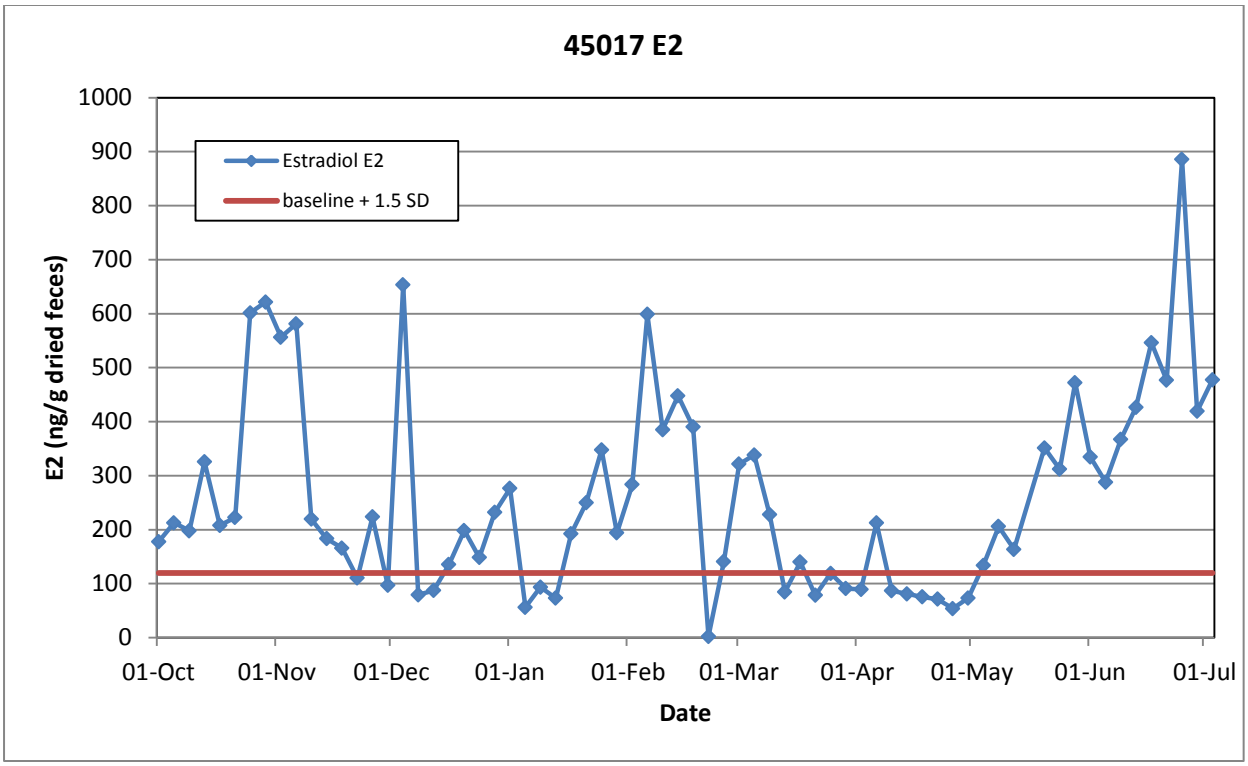

45016 T
2500
2000
1500
1000
500
0
T (ng/g dried feces)
Testosterone T
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

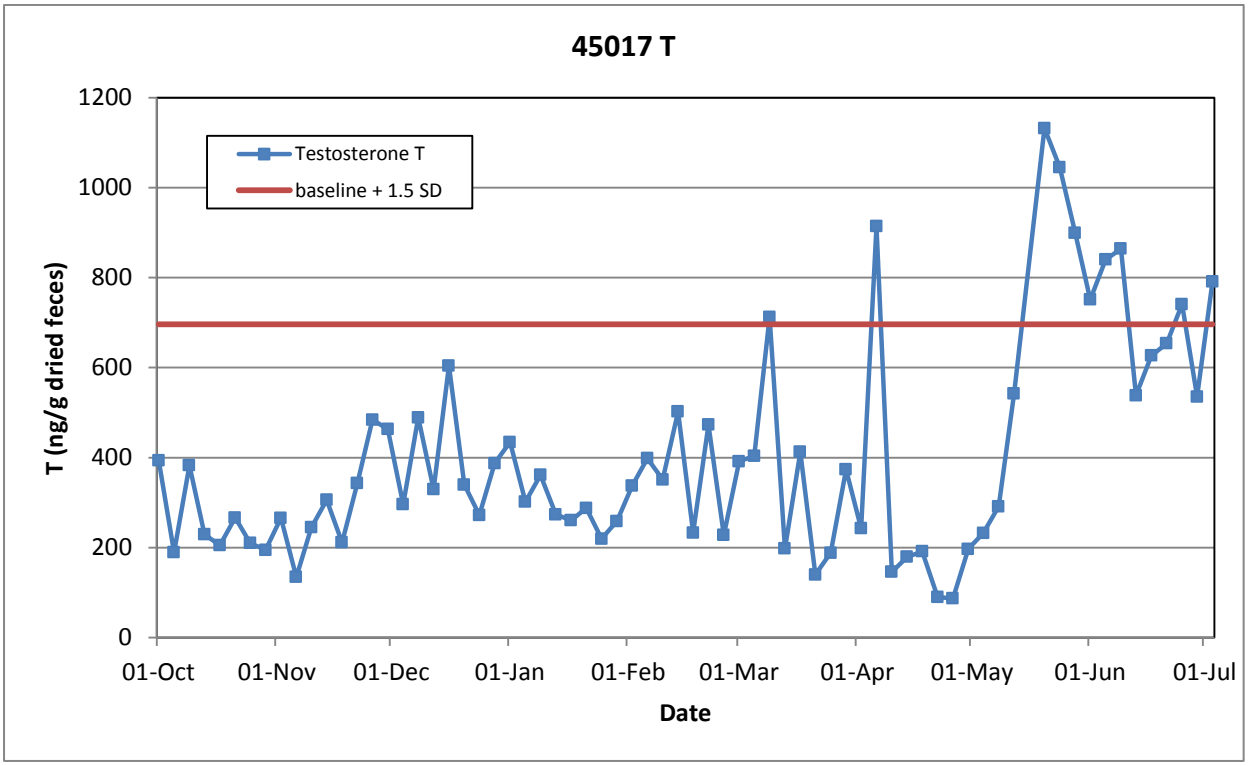

45017
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T, CORT (ng/g dried feces)
P (ng/g dried feces)
0
200
400
600
800
1000
1200
0
2000
4000
6000
8000
10000
12000
1-Oct
1-Nov
1-Dec
1-Jan
1-Feb
1-Mar
1-Apr
1-May
1-Jun
1-Jul
Date

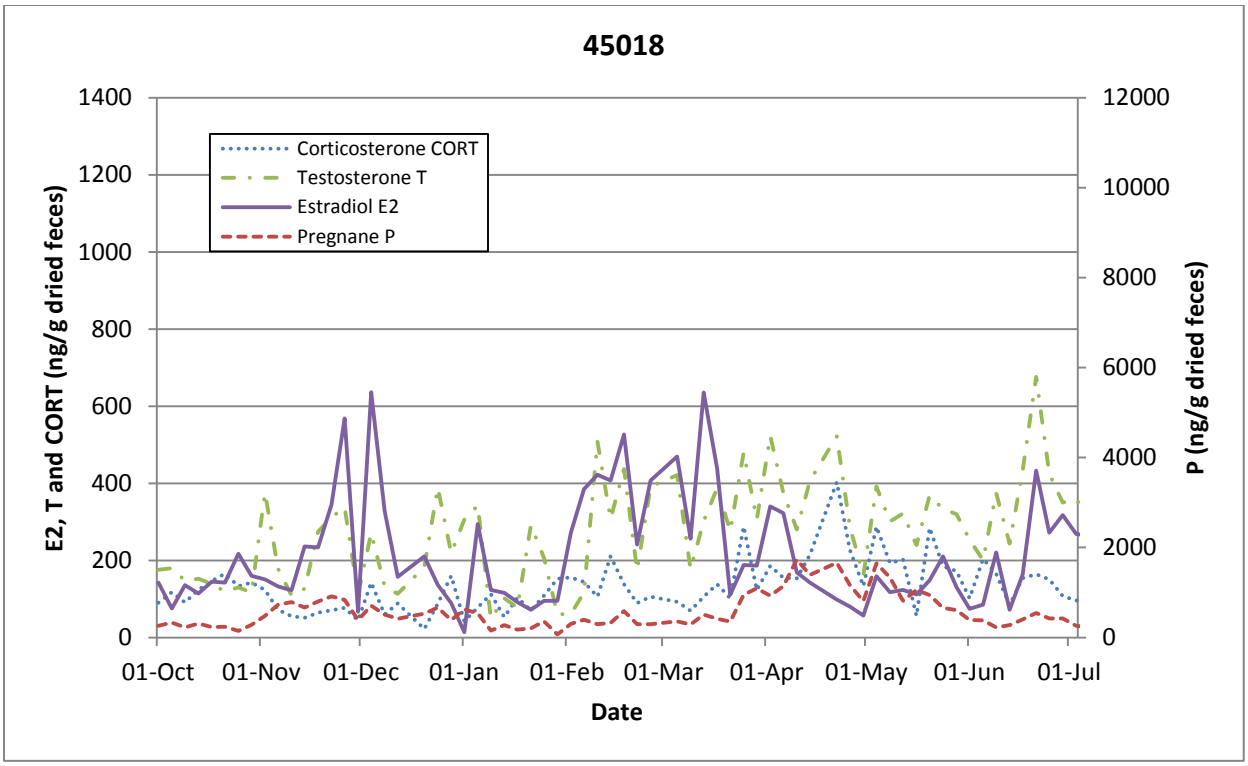

45017 Follicular changes
Length L
Width W
Surface Area SA
L & W (cm) and SA (cm²)
0
0.2
0.4
0.6
0.8
1
1.2
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

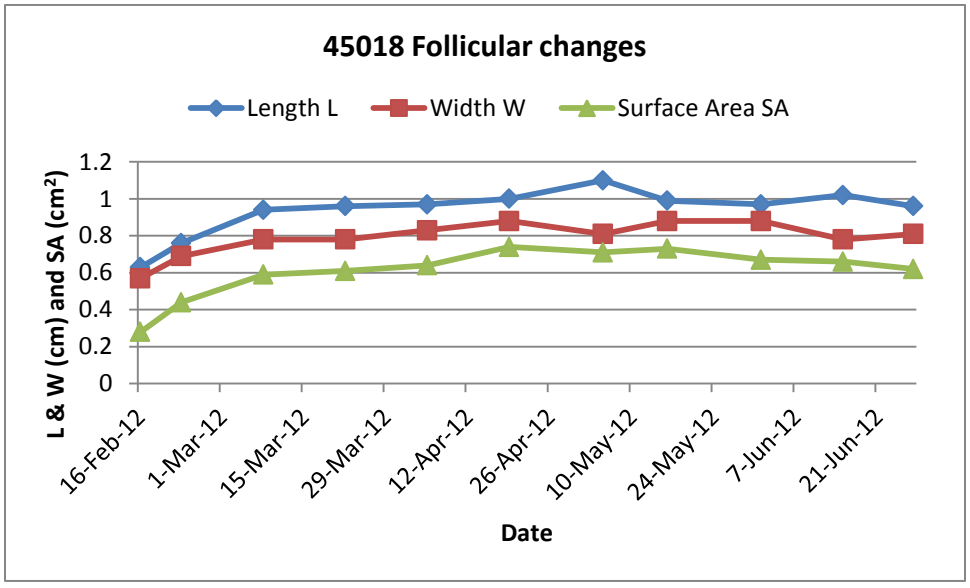

45017 CORT
Corticosterone CORT
baseline + 1.5 SD
CORT (ng/g dried feces)
300
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

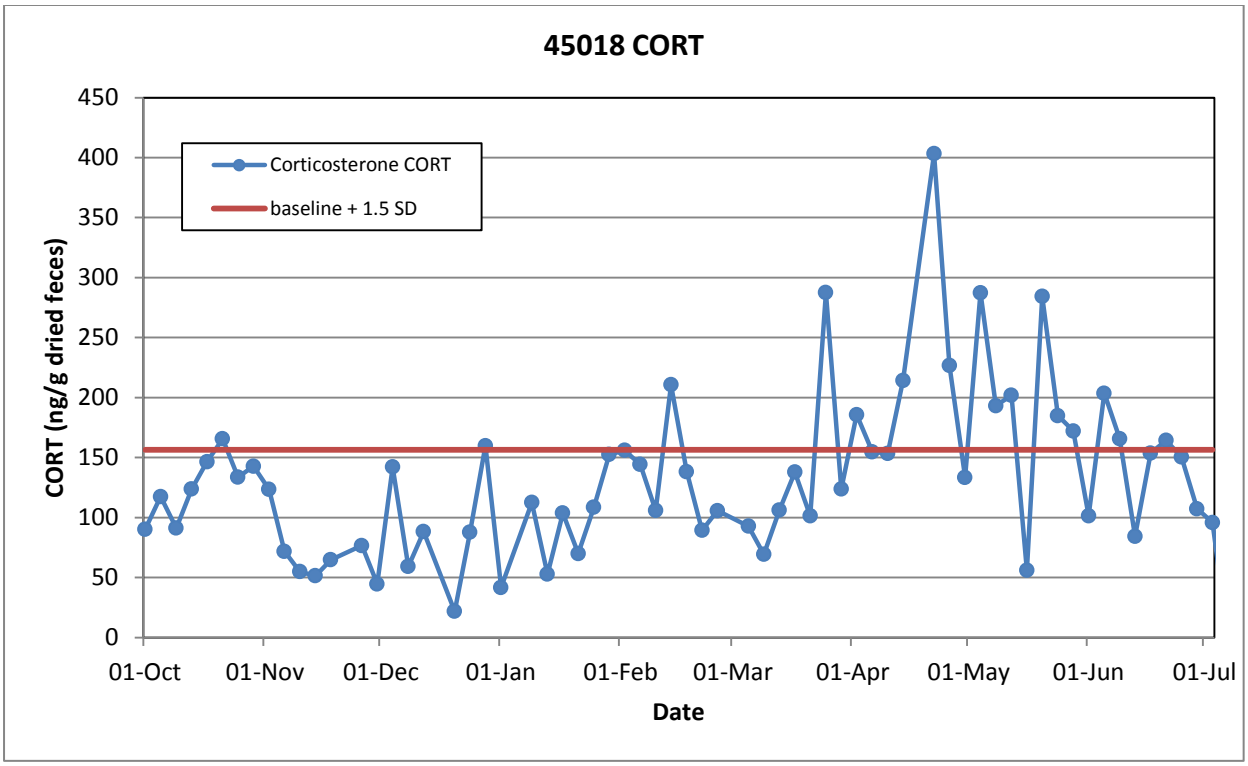

45017 P
Pregnane P
baseline + 1.5 SD
P (ng/g dried feces)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

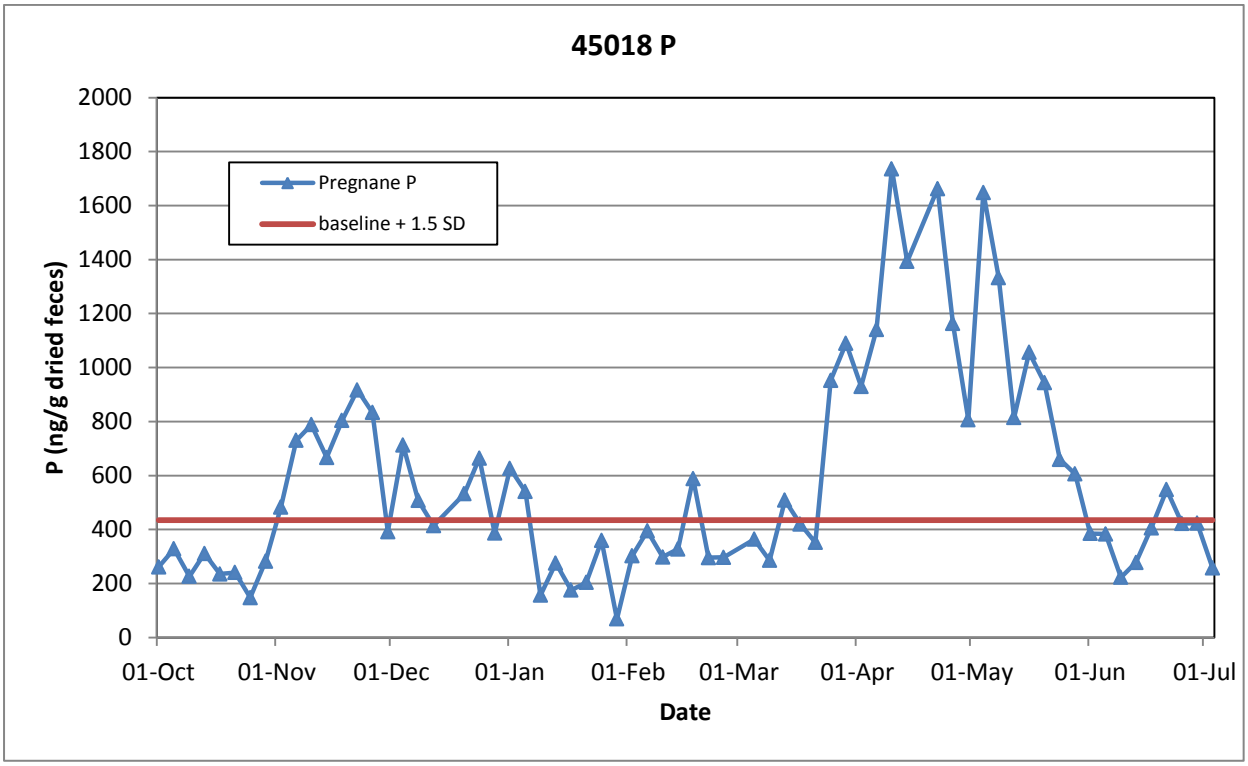

45017 E2
Estradiol E2
baseline + 1.5 SD
E2 (ng/g dried feces)
1000
900
800
700
600
500
400
300
200
100
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

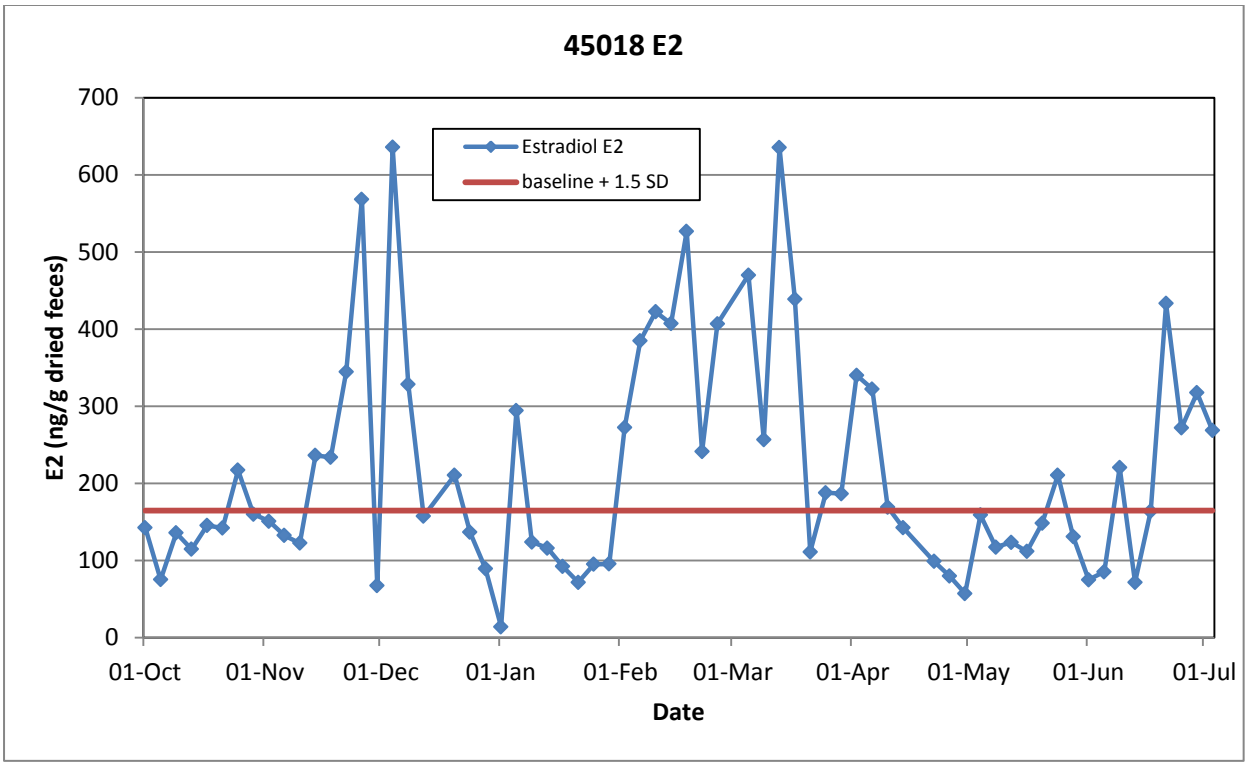

45017 T
Testosterone T
baseline + 1.5 SD
T (ng/g dried feces)
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

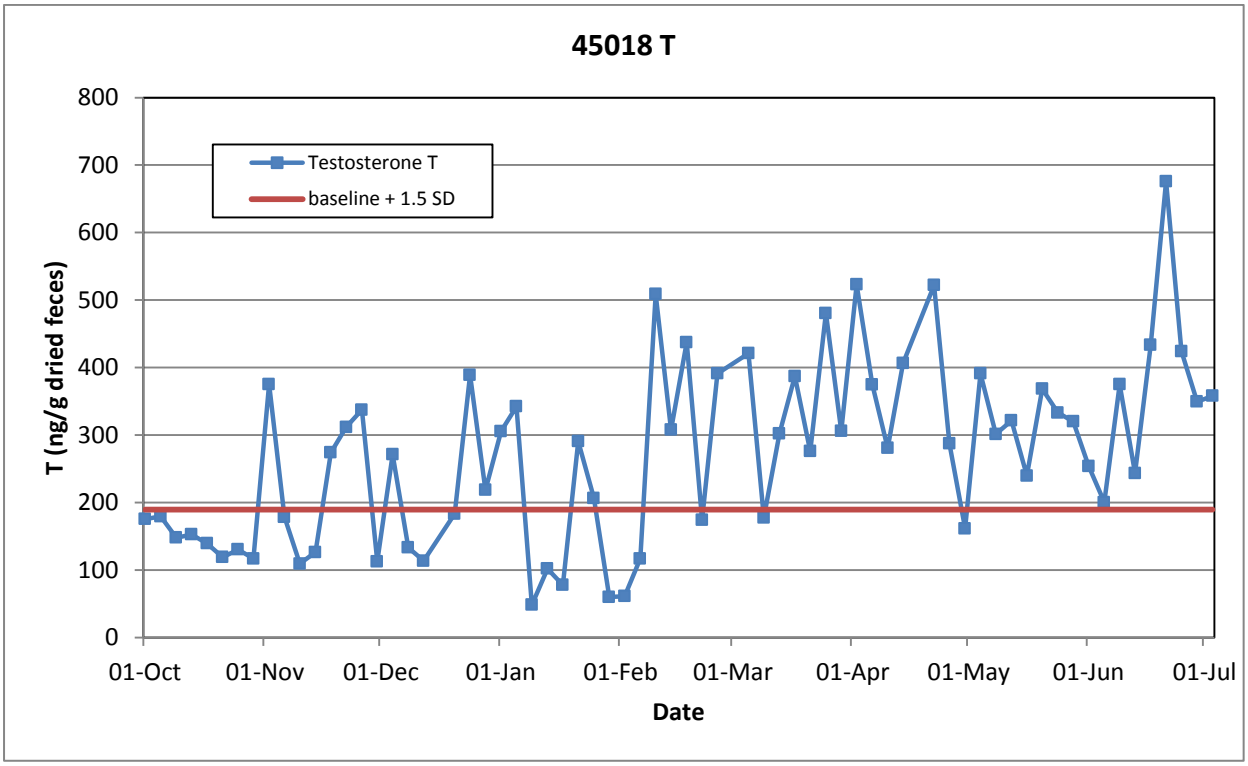

45018
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
1400
1200
1000
800
600
400
200
0
12000
10000
8000
6000
4000
2000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

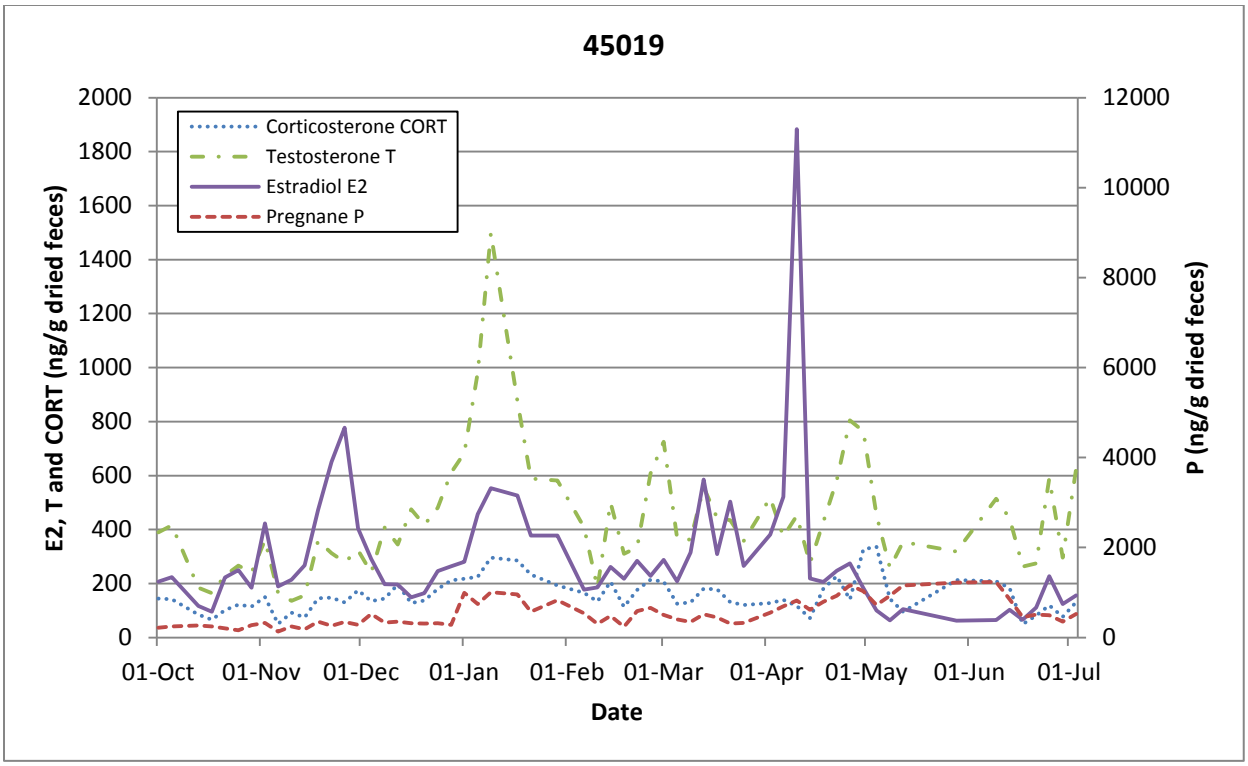

45018 Follicular changes
Length L
Width W
Surface Area SA
L & W (cm) and SA (cm²)
1.2
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

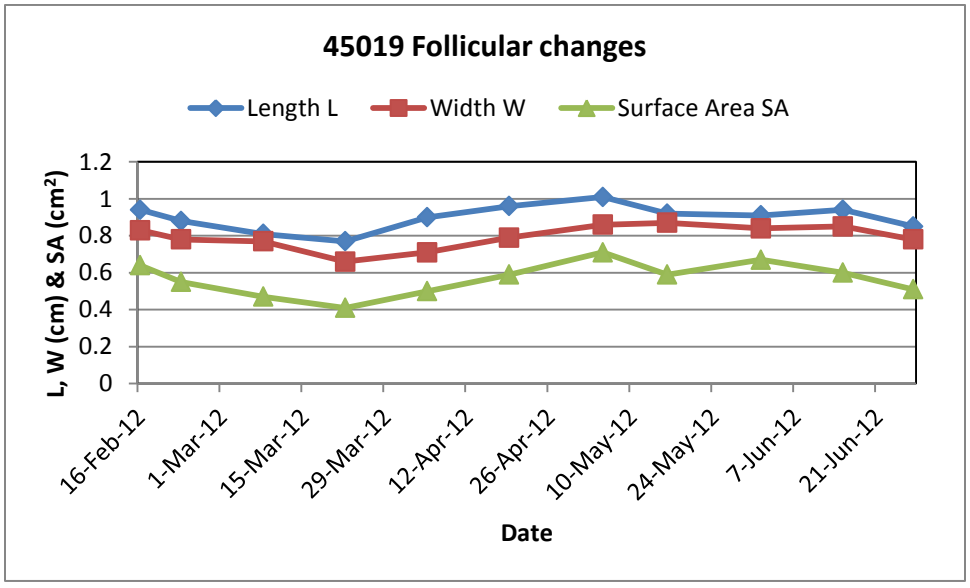

45018 CORT
Corticosterone CORT
baseline + 1.5 SD
CORT (ng/g dried feces)
450
400
350
300
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

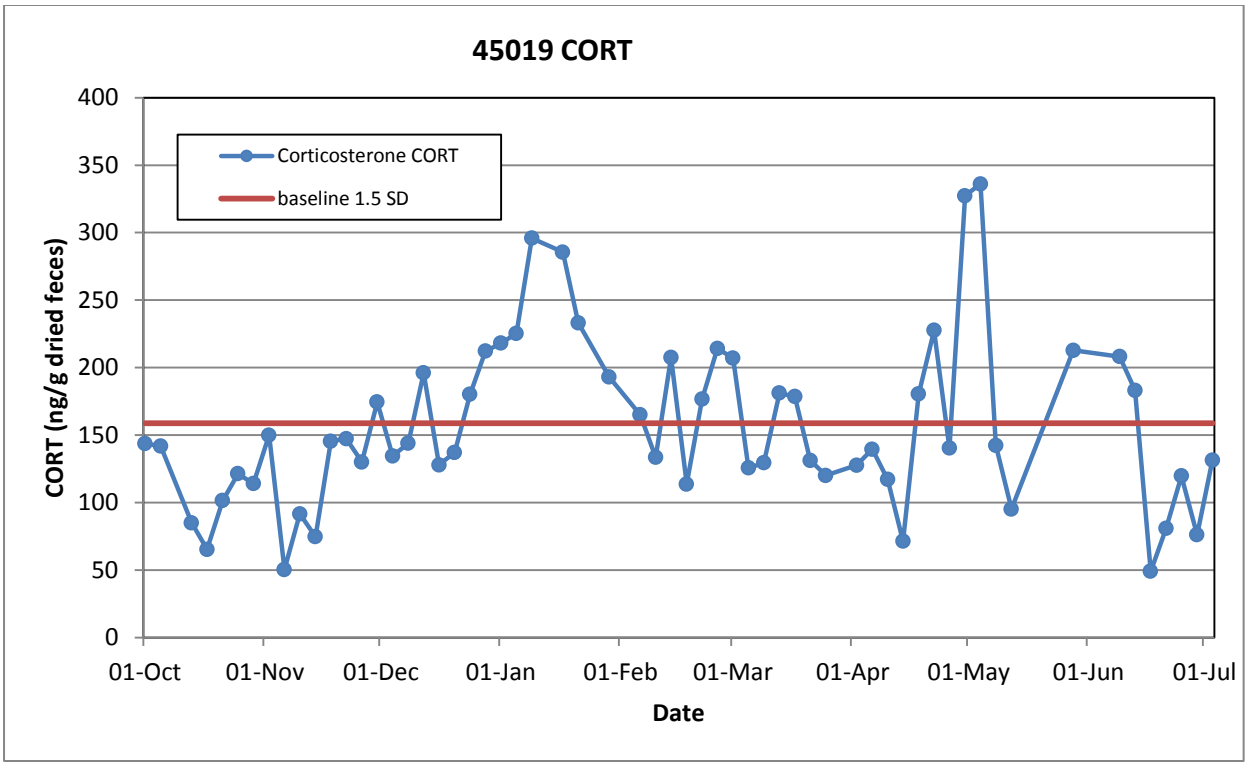

45018 P
Pregnane P
baseline + 1.5 SD
P (ng/g dried feces)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

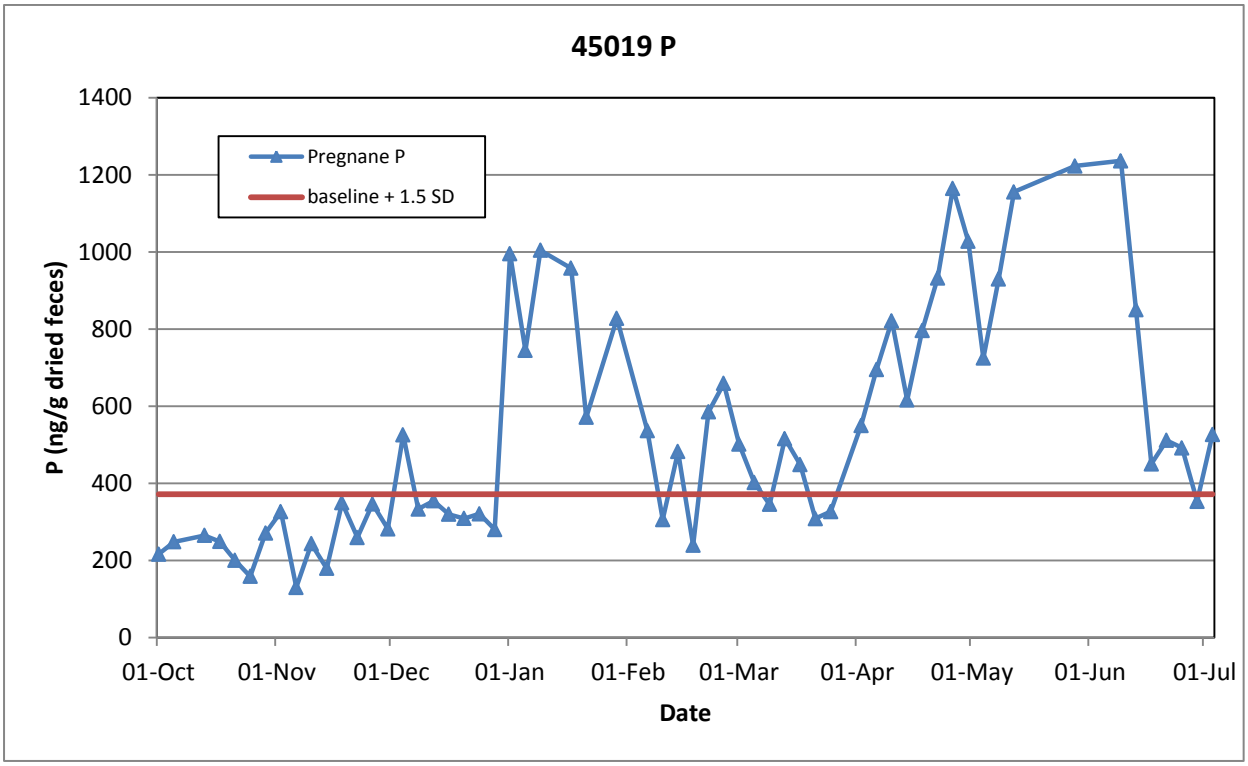

45018 E2
Estradiol E2
baseline + 1.5 SD
E2 (ng/g dried feces)
0
100
200
300
400
500
600
700
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

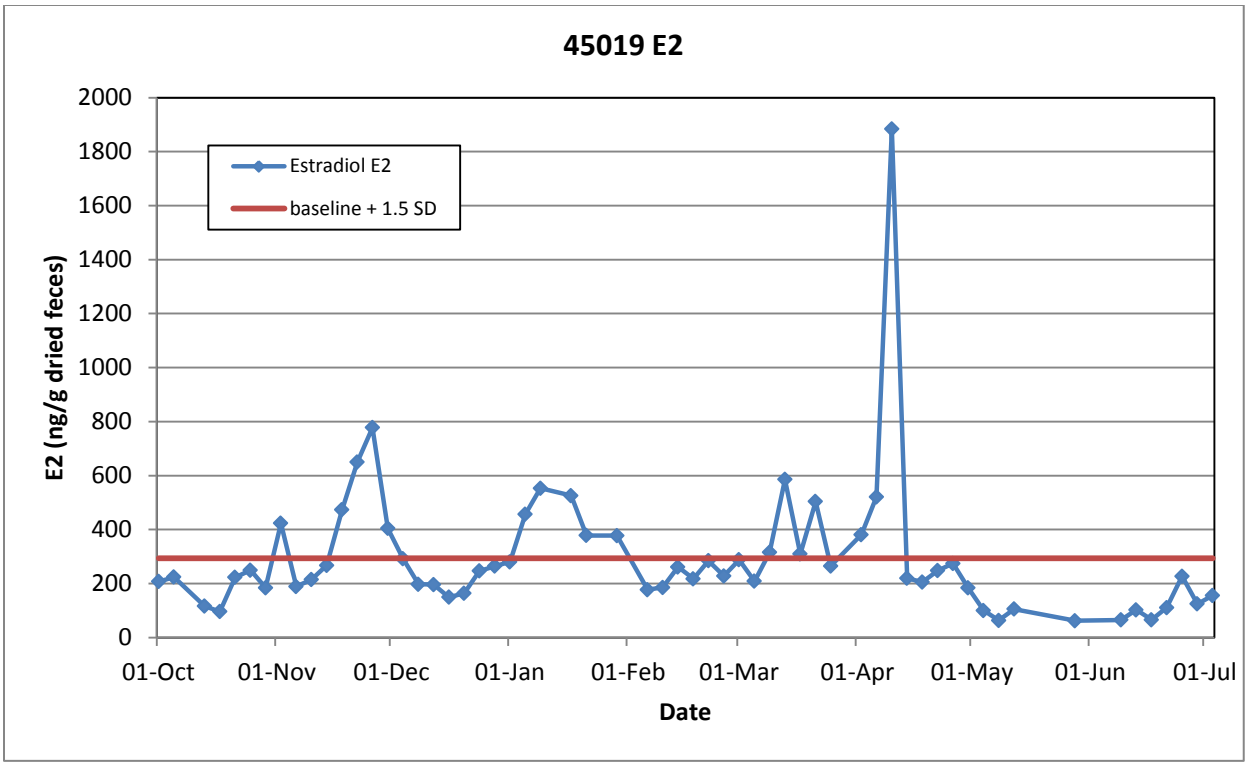

45018 T
Testosterone T
baseline + 1.5 SD
T (ng/g dried feces)
0
100
200
300
400
500
600
700
800
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

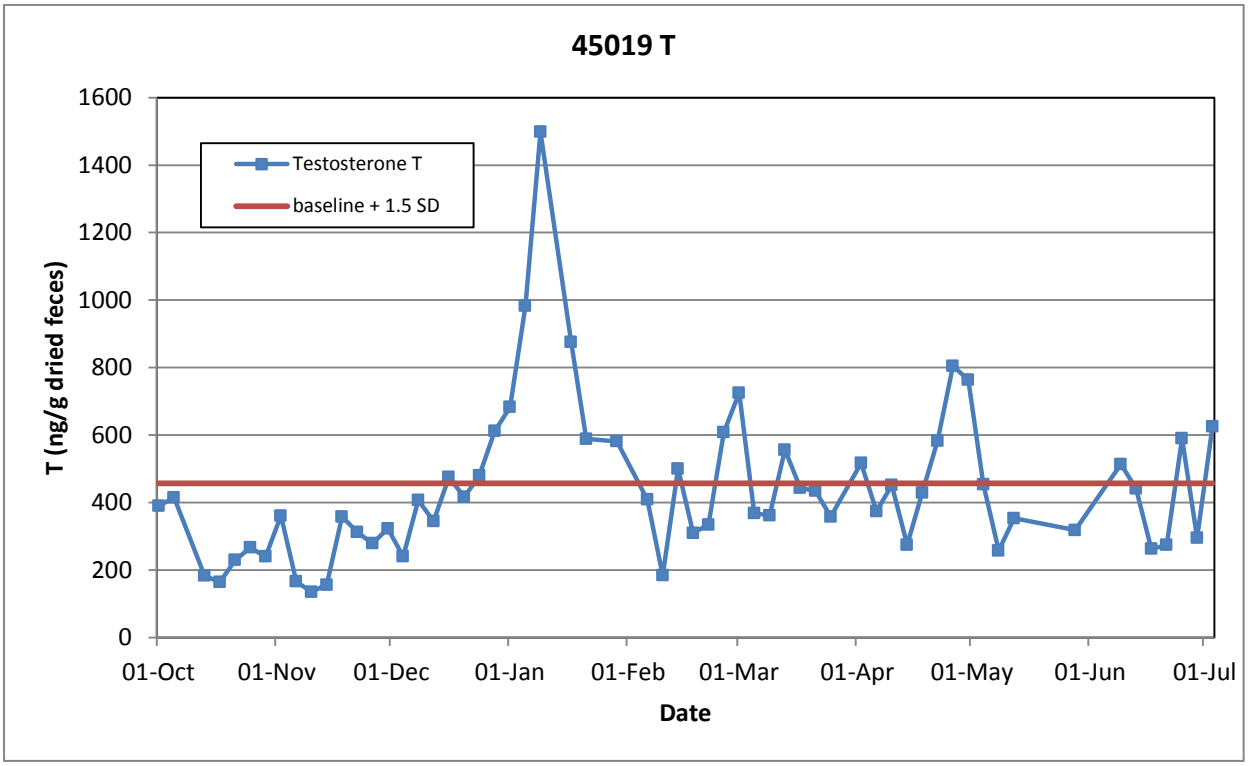

45019
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
12000
10000
8000
6000
4000
2000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

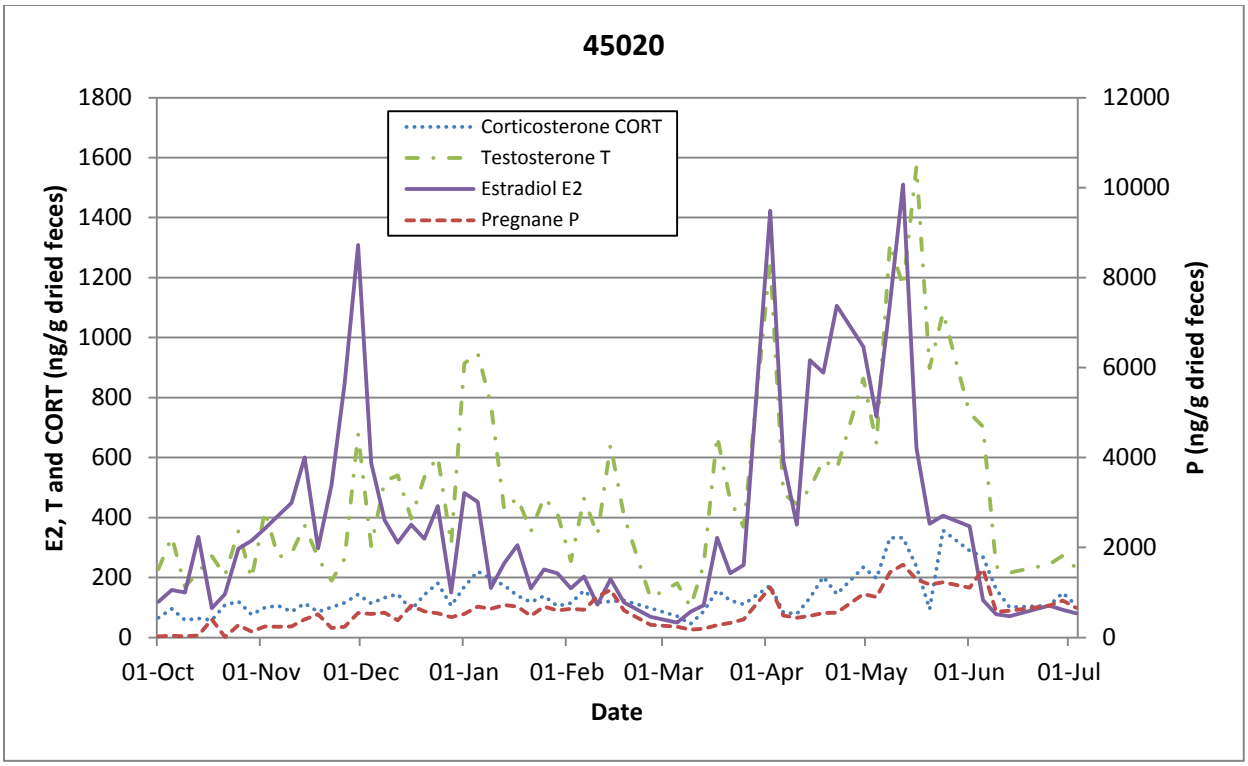

45019 Follicular changes
Length L
Width W
Surface Area SA
L, W (cm) & SA (cm²)
1.2
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

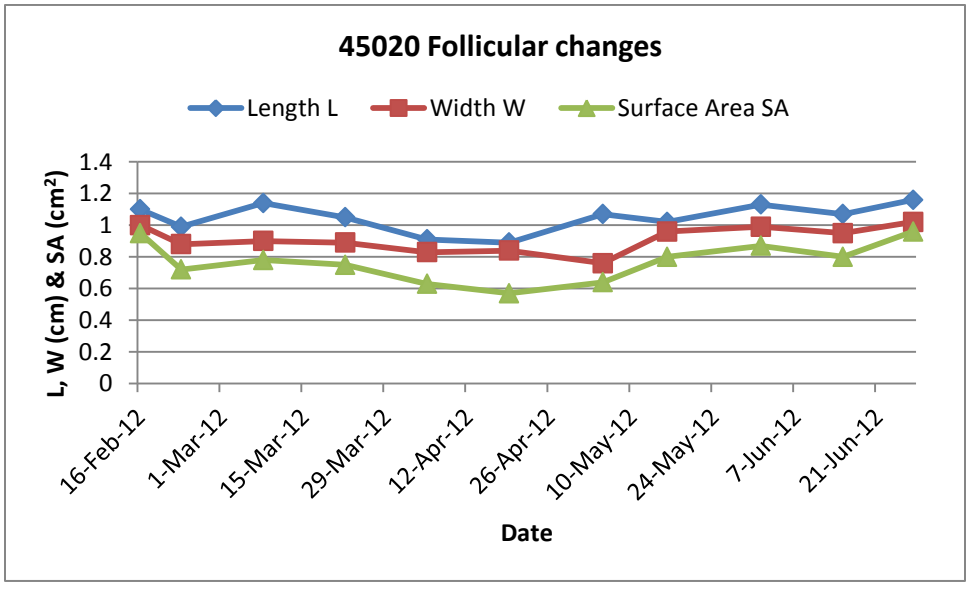

45019 CORT
Corticosterone CORT
baseline 1.5 SD
CORT (ng/g dried feces)
400
350
300
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

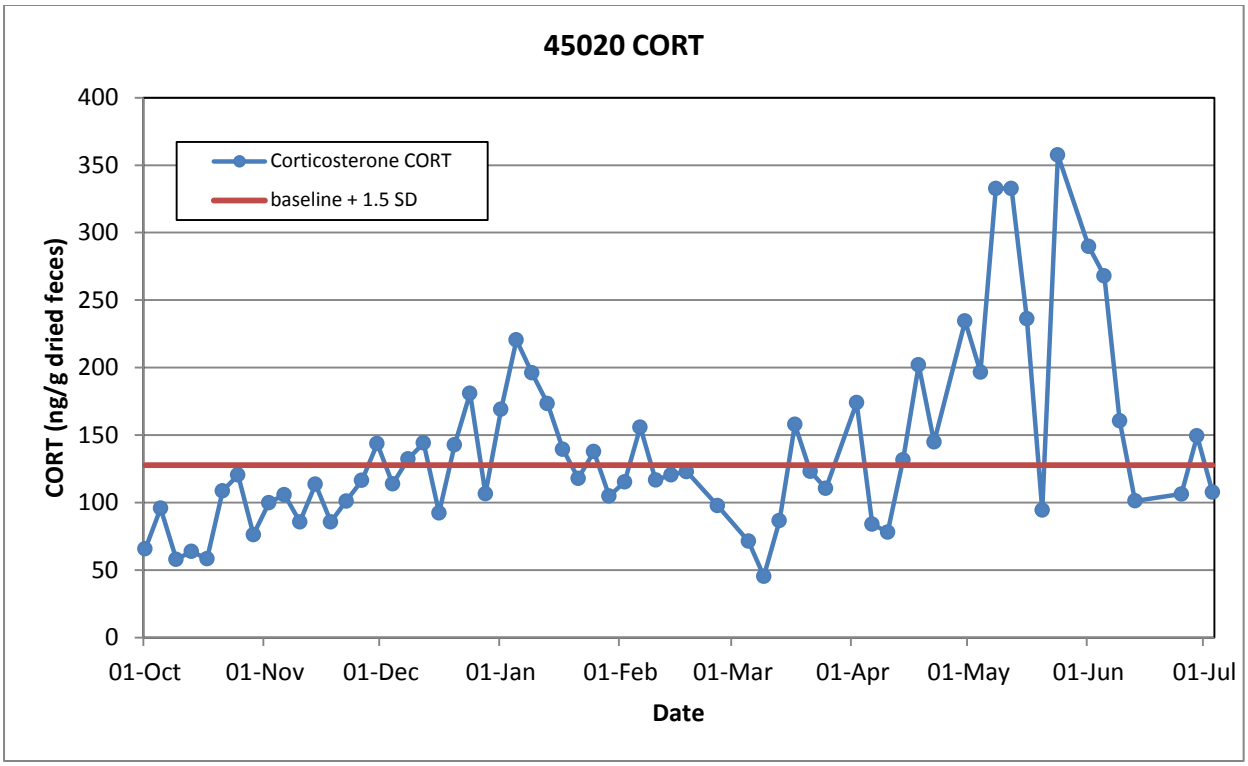

45019 P
Pregnane P
baseline + 1.5 SD
P (ng/g dried feces)
1400
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

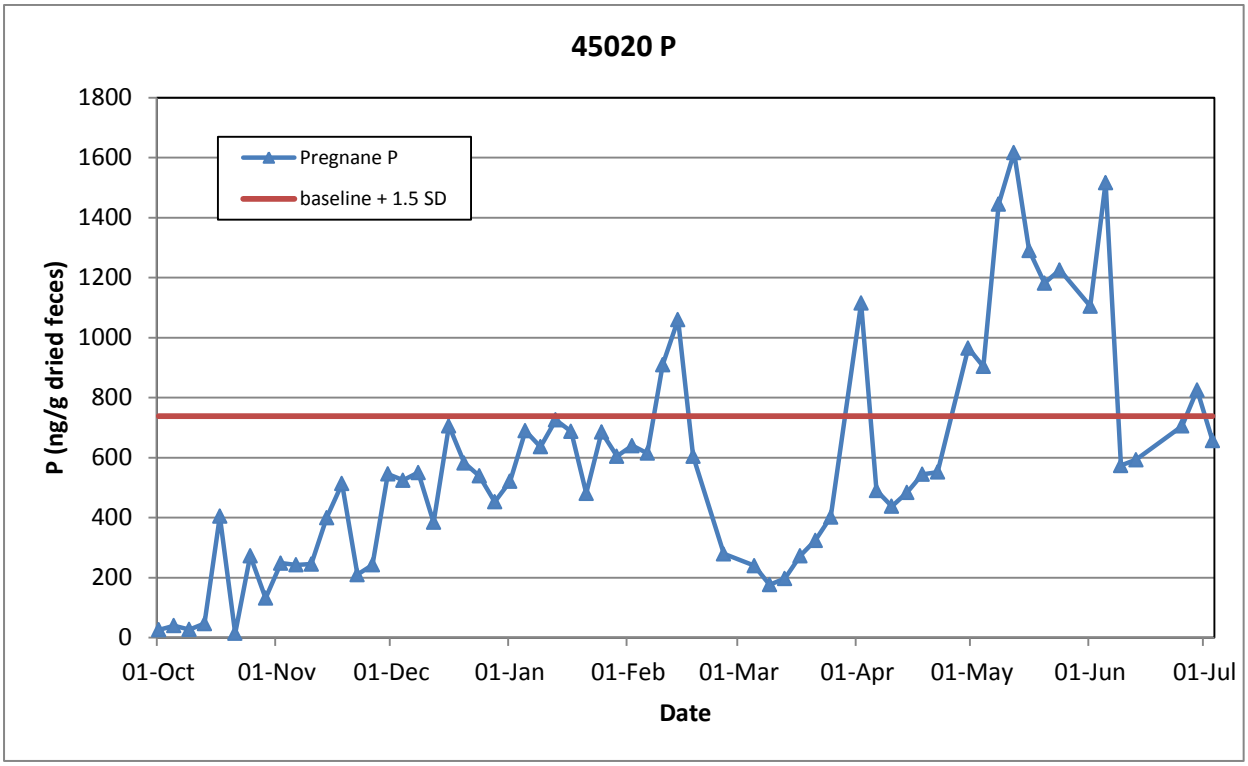

45019 E2
Estradiol E2
baseline + 1.5 SD
E2 (ng/g dried feces)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

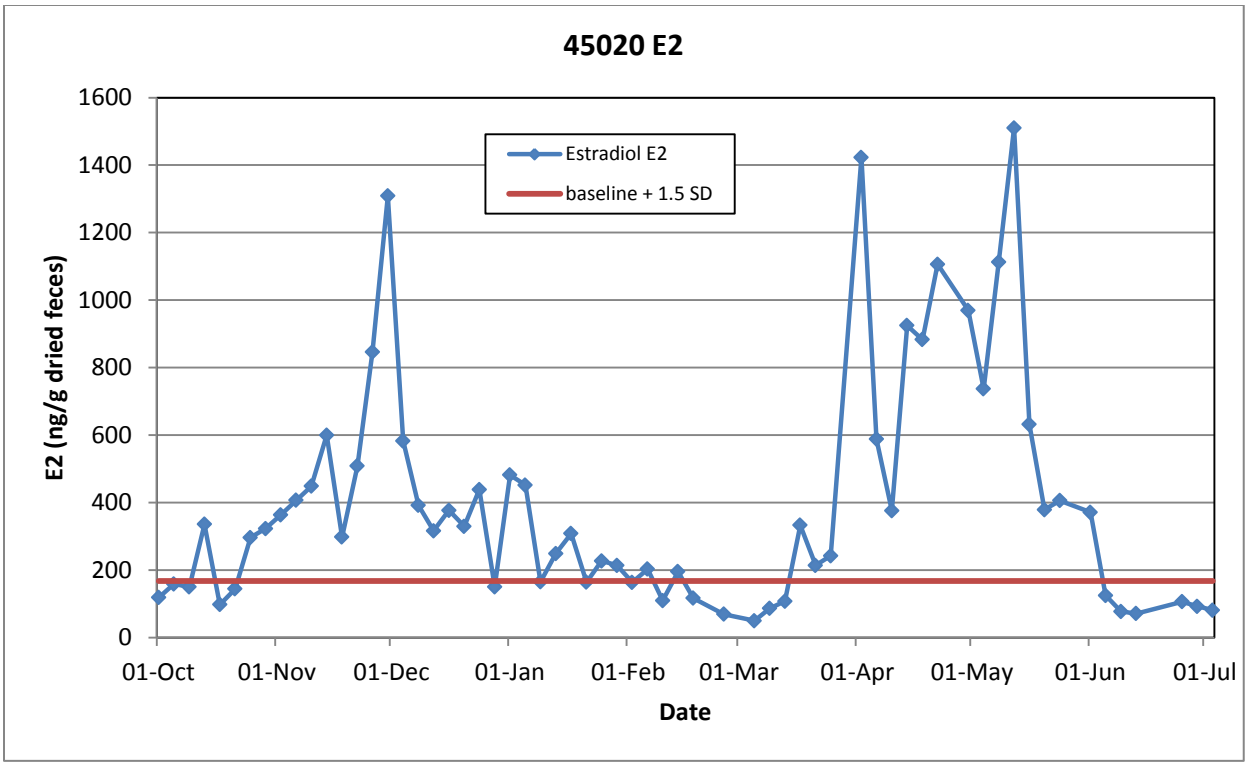

45019 T
Testosterone T
baseline + 1.5 SD
T (ng/g dried feces)
1600
1400
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

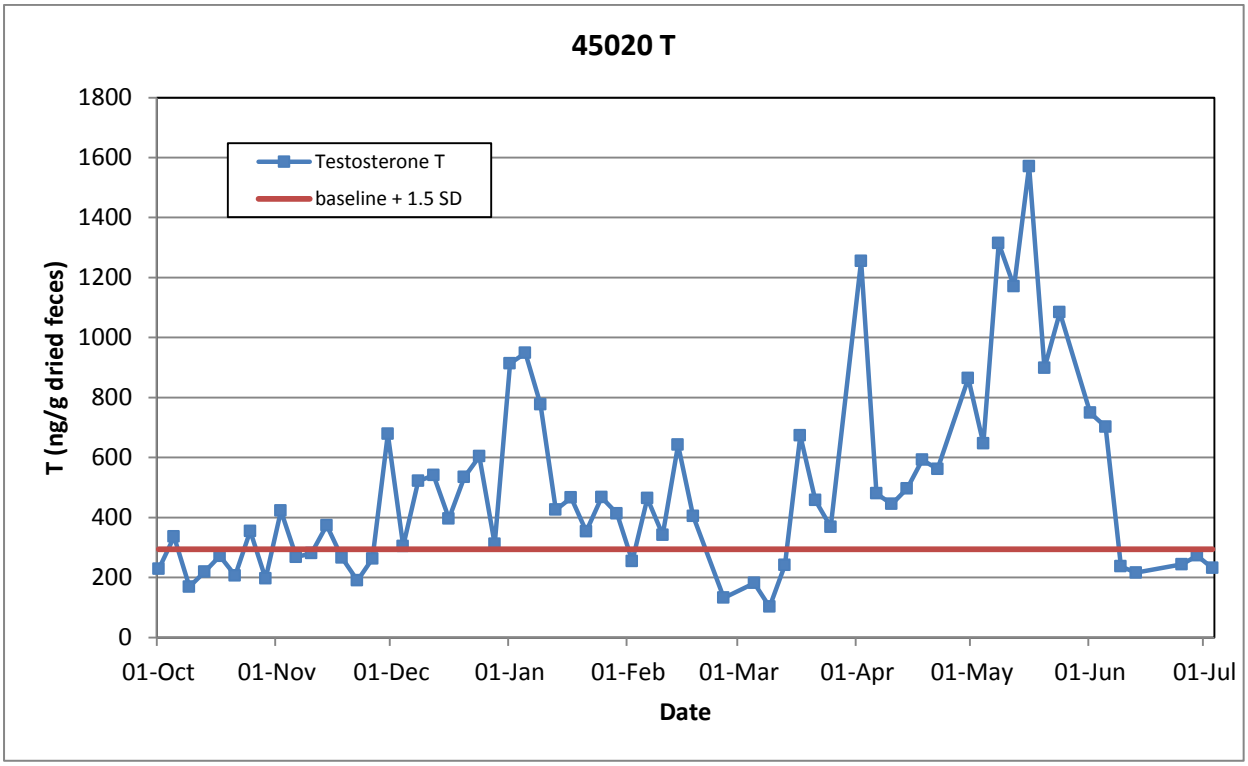

45020
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
1800
1600
1400
1200
1000
800
600
400
200
0
12000
10000
8000
6000
4000
2000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

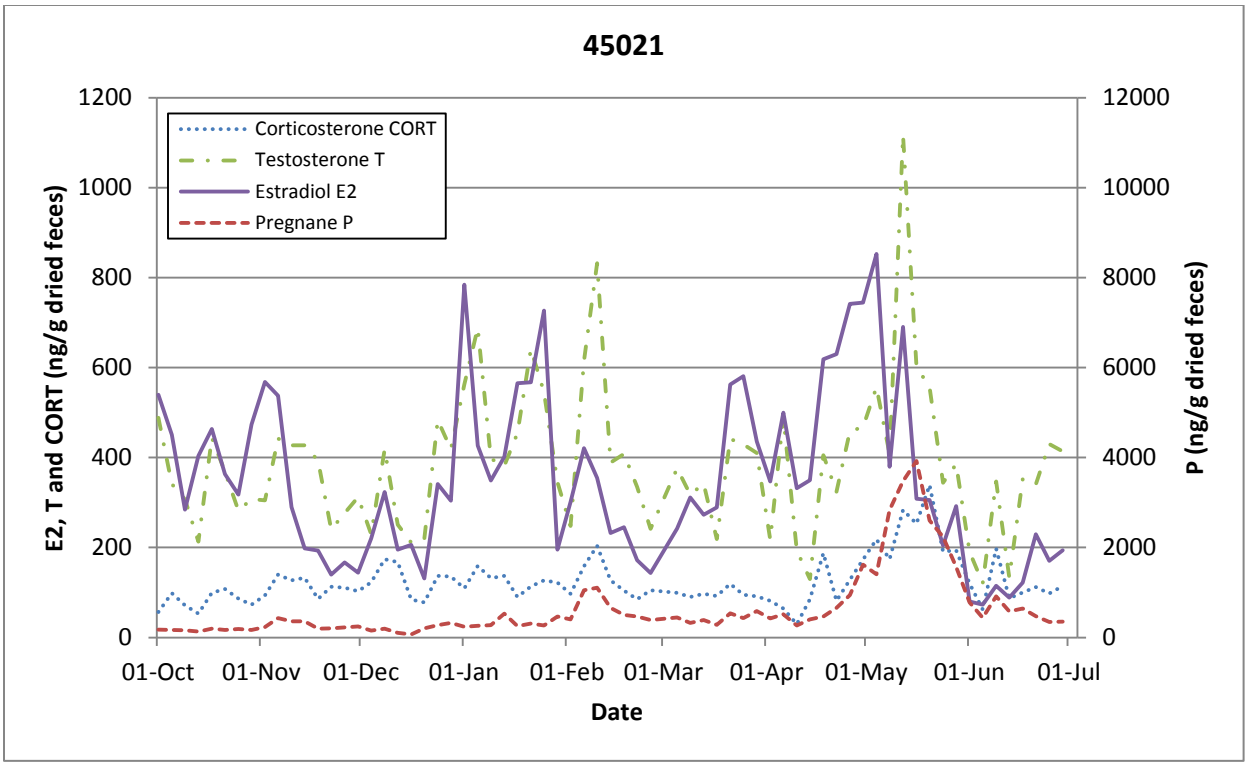

45020 Follicular changes
Length L
Width W
Surface Area SA
L, W (cm) & SA (cm²)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

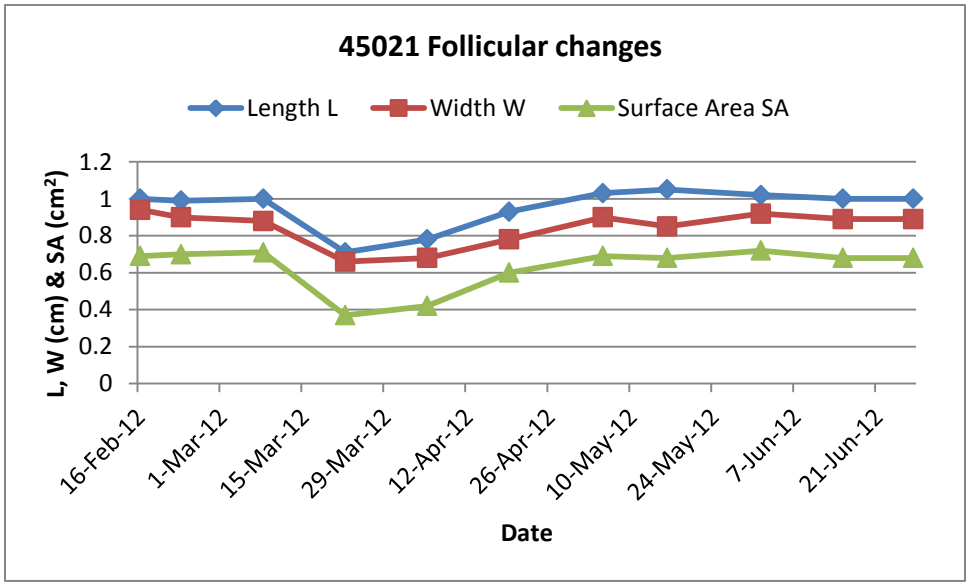

45020 CORT
Corticosterone CORT
baseline + 1.5 SD
CORT (ng/g dried feces)
400
350
300
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

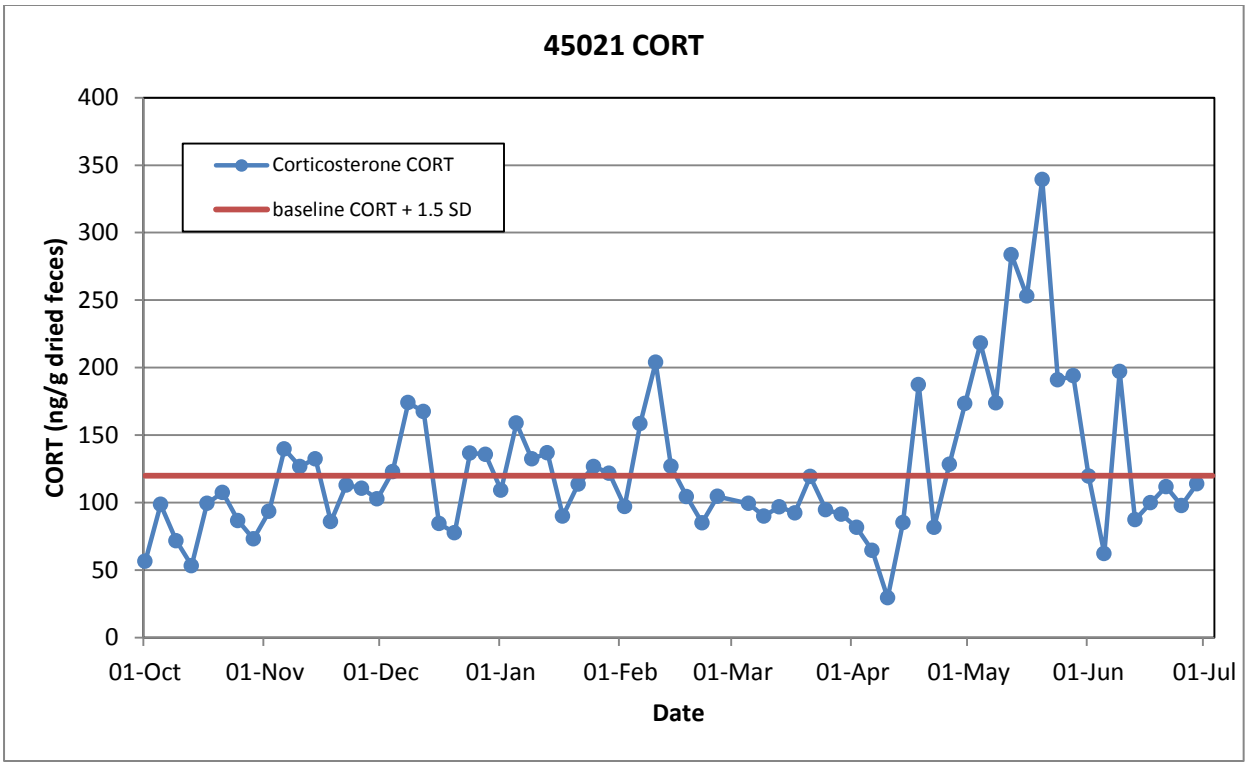

45020 P
Pregnane P
baseline + 1.5 SD
P (ng/g dried feces)
1800
1600
1400
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

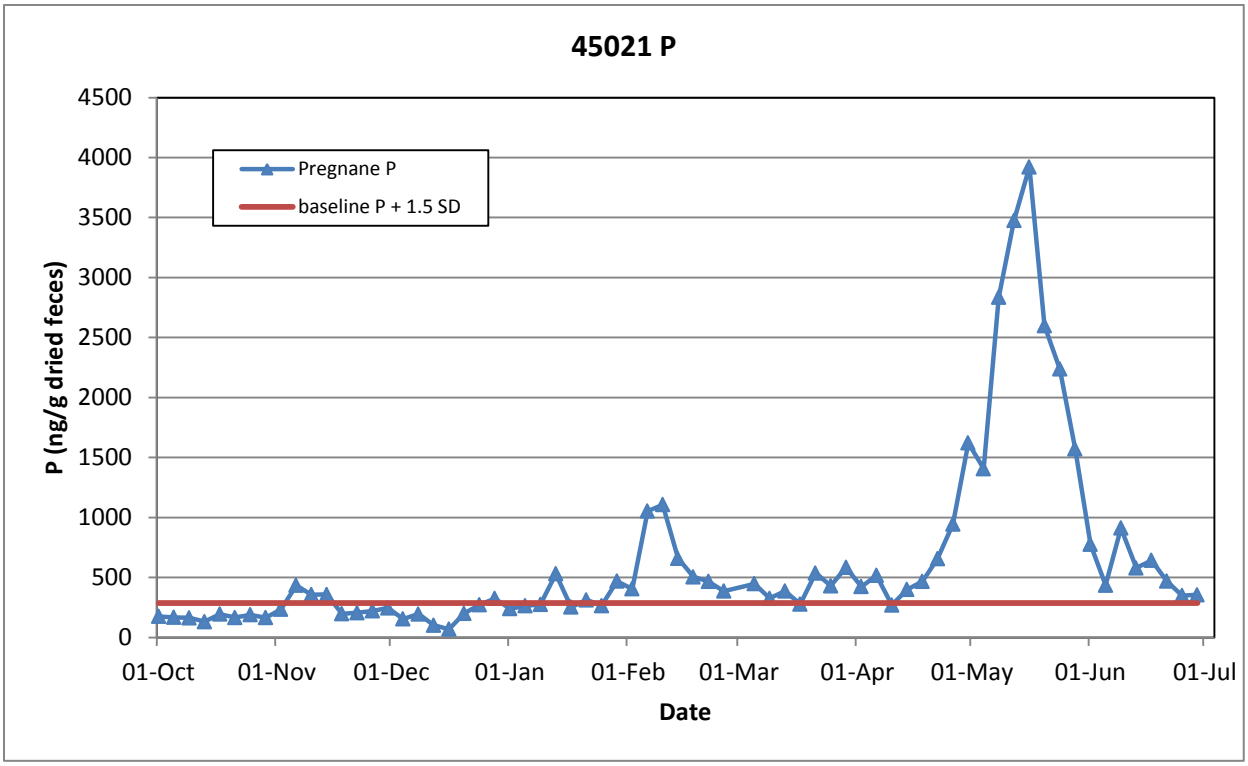

45020 E2
Estradiol E2
baseline + 1.5 SD
E2 (ng/g dried feces)
1600
1400
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

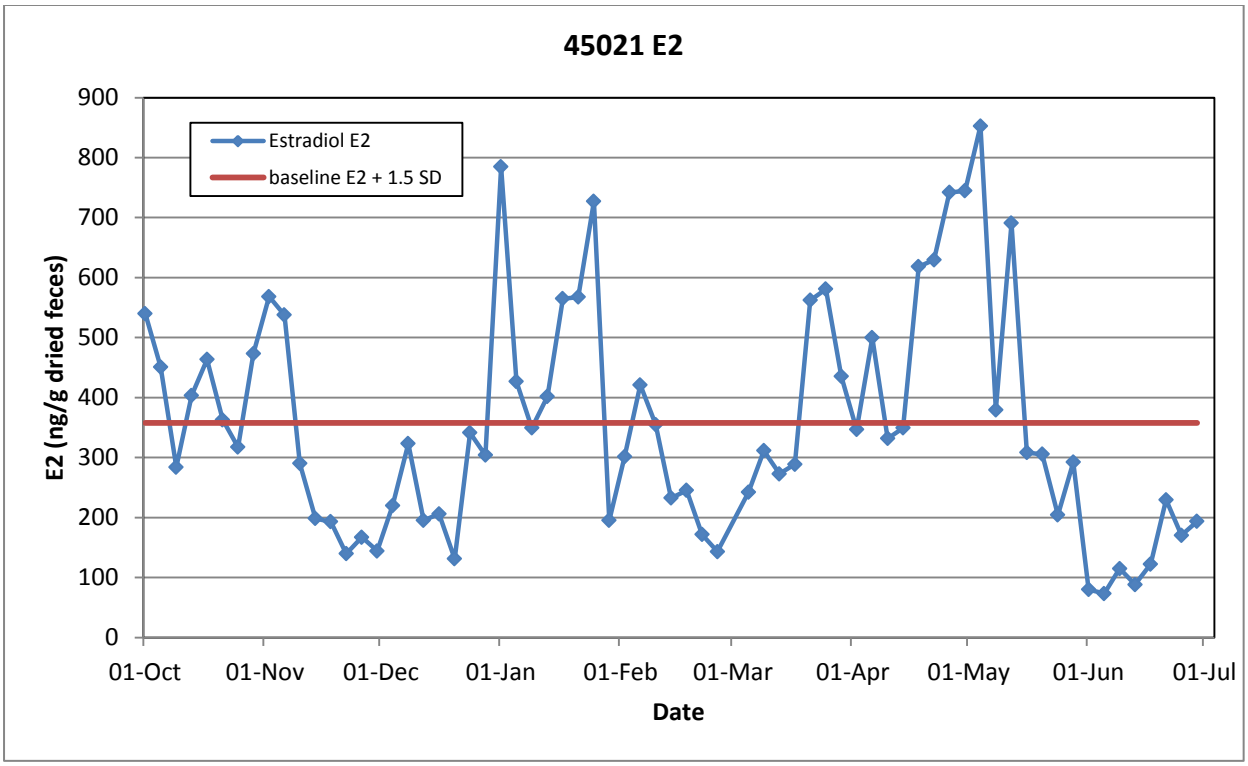

45020 T
Testosterone T
baseline + 1.5 SD
T (ng/g dried feces)
1800
1600
1400
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

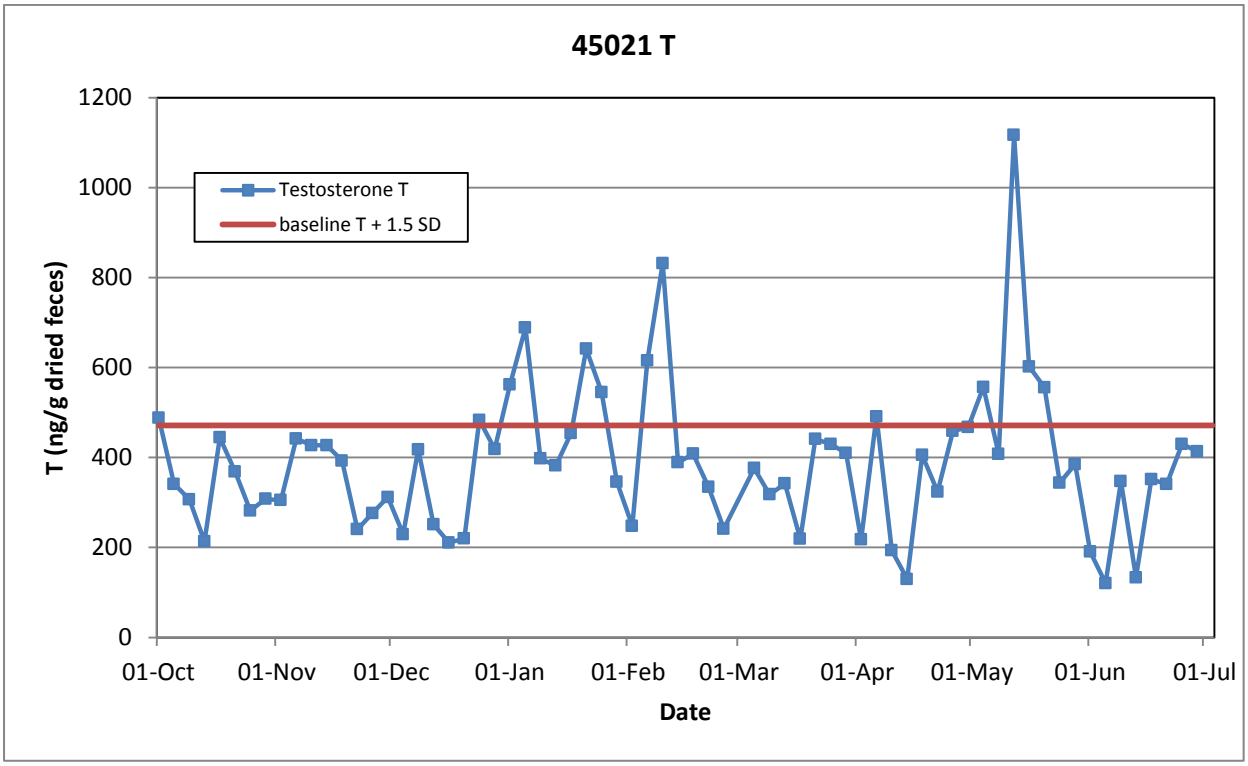

45021
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
1200
1000
800
600
400
200
0
12000
10000
8000
6000
4000
2000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

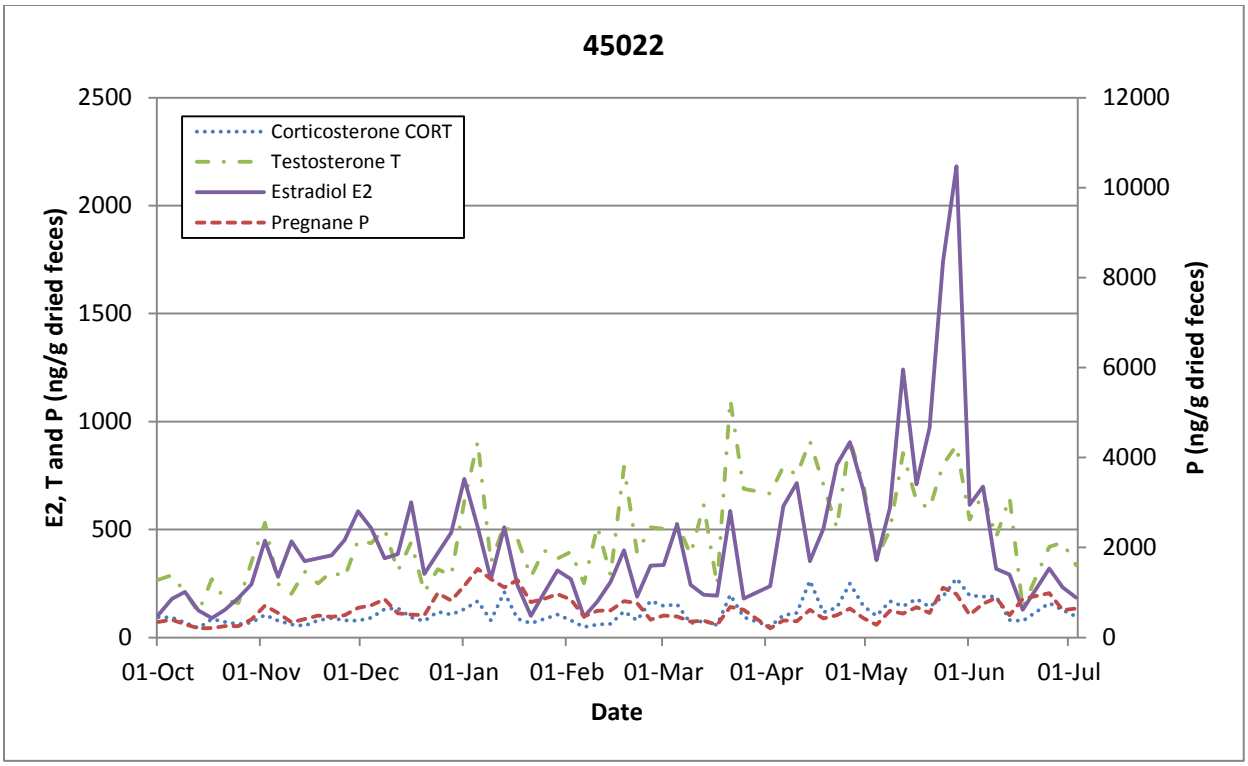

45021 Follicular changes
Length L
Width W
Surface Area SA
L, W (cm) & SA (cm²)
1.2
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

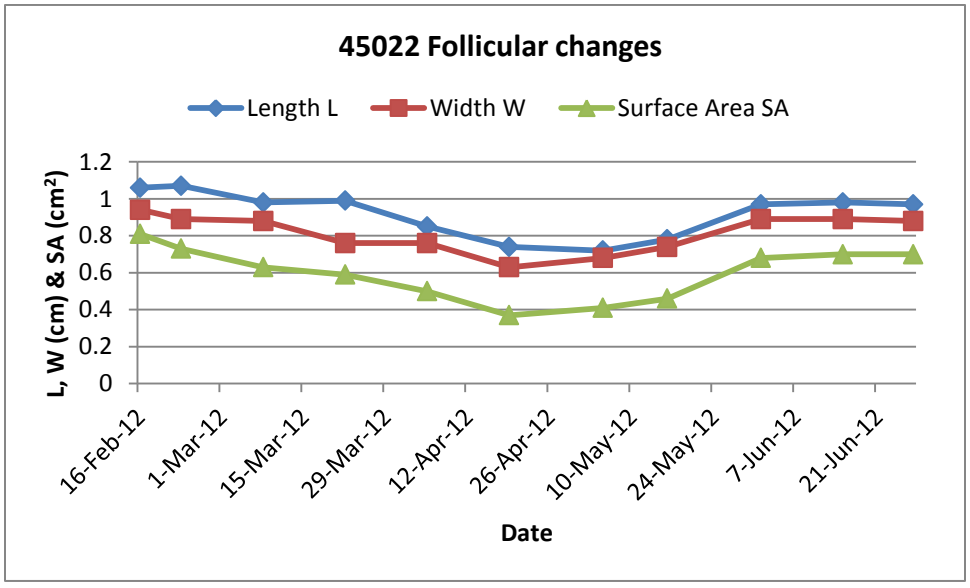

45021 CORT
Corticosterone CORT
baseline CORT + 1.5 SD
CORT (ng/g dried feces)
400
350
300
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

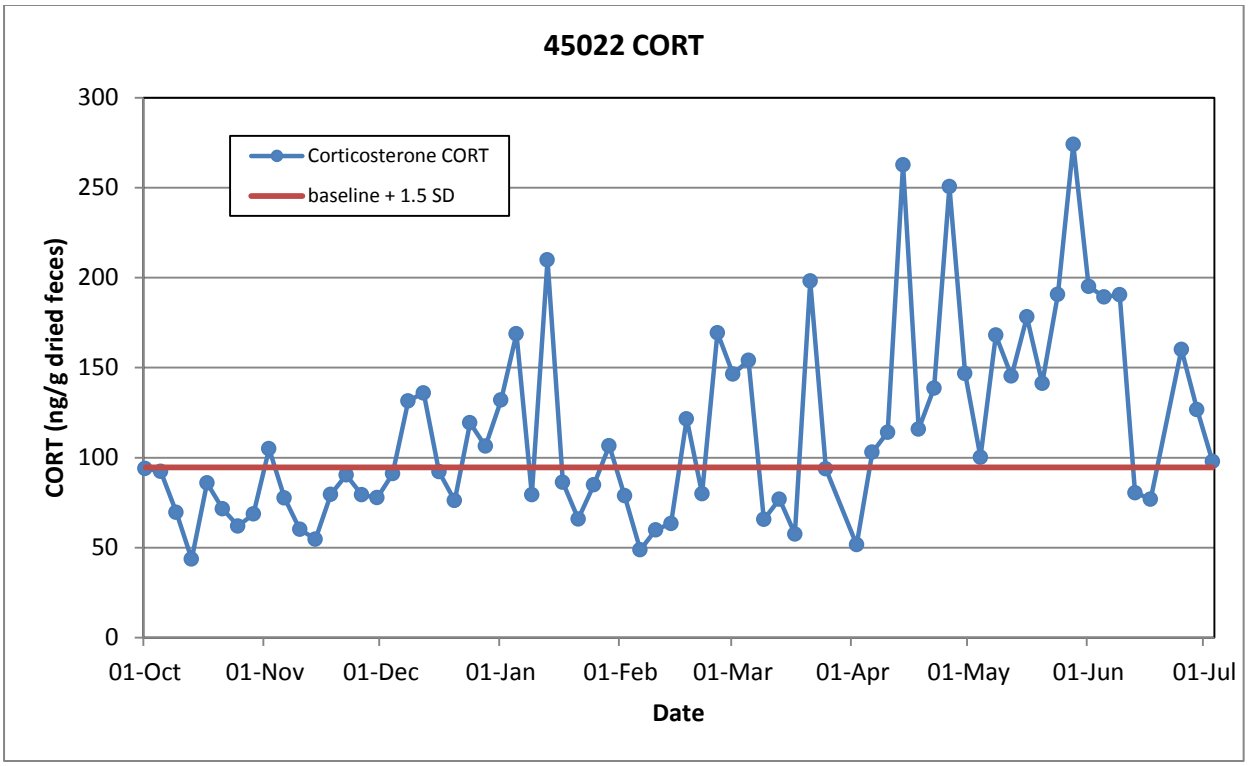

45021 P
Pregnane P
baseline P + 1.5 SD
P (ng/g dried feces)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

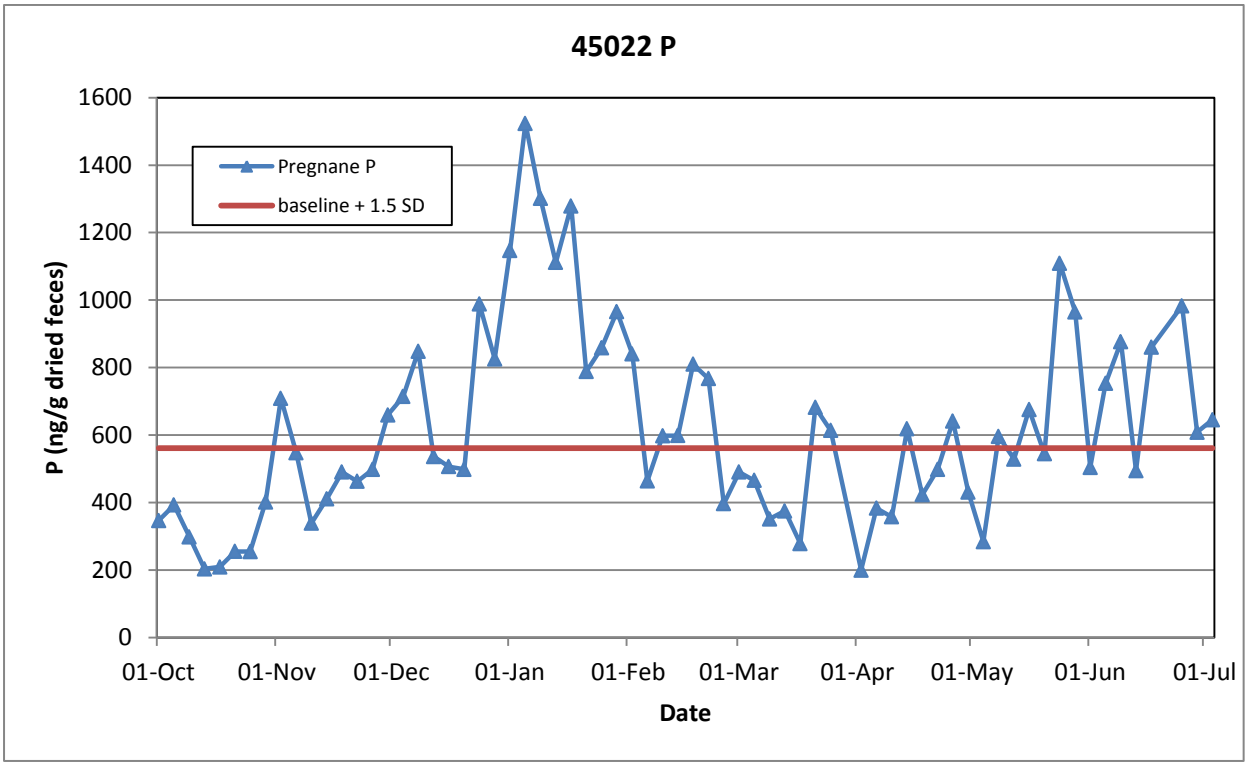

45021 E2
Estradiol E2
baseline E2 + 1.5 SD
E2 (ng/g dried feces)
900
800
700
600
500
400
300
200
100
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

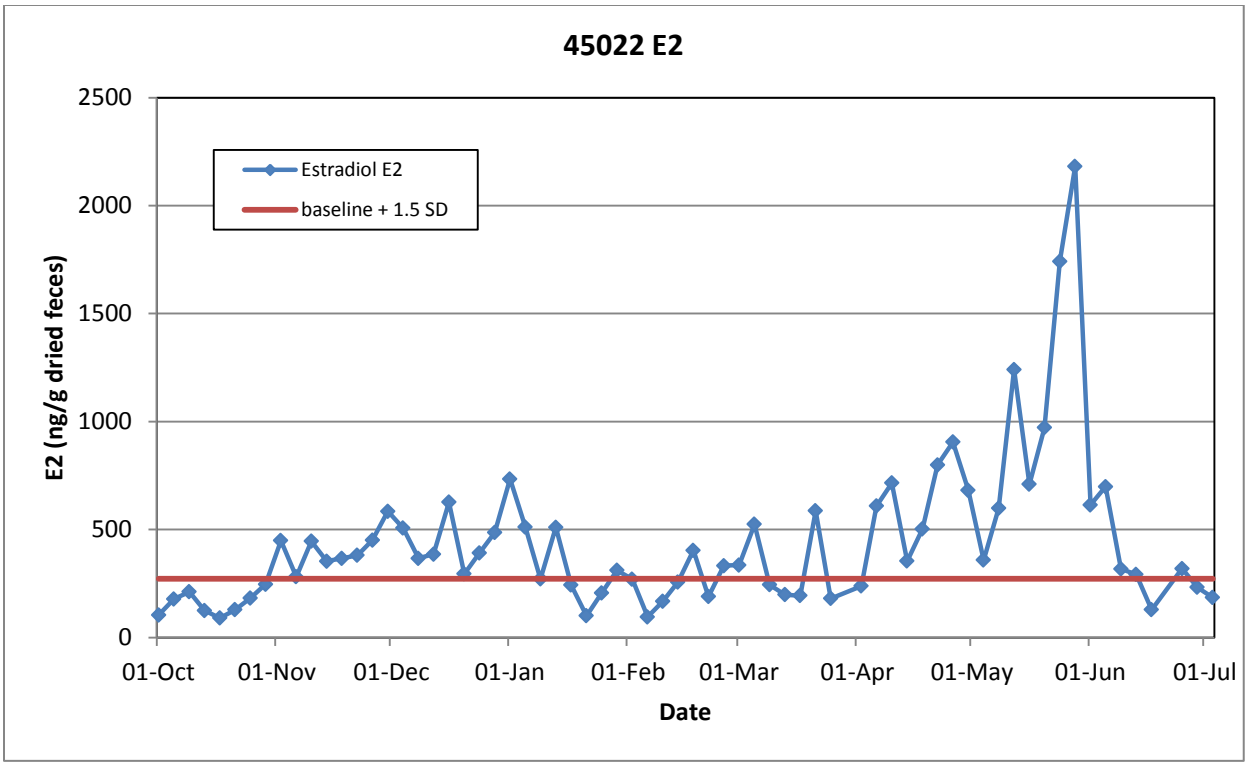

45021 T
Testosterone T
baseline T + 1.5 SD
T (ng/g dried feces)
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

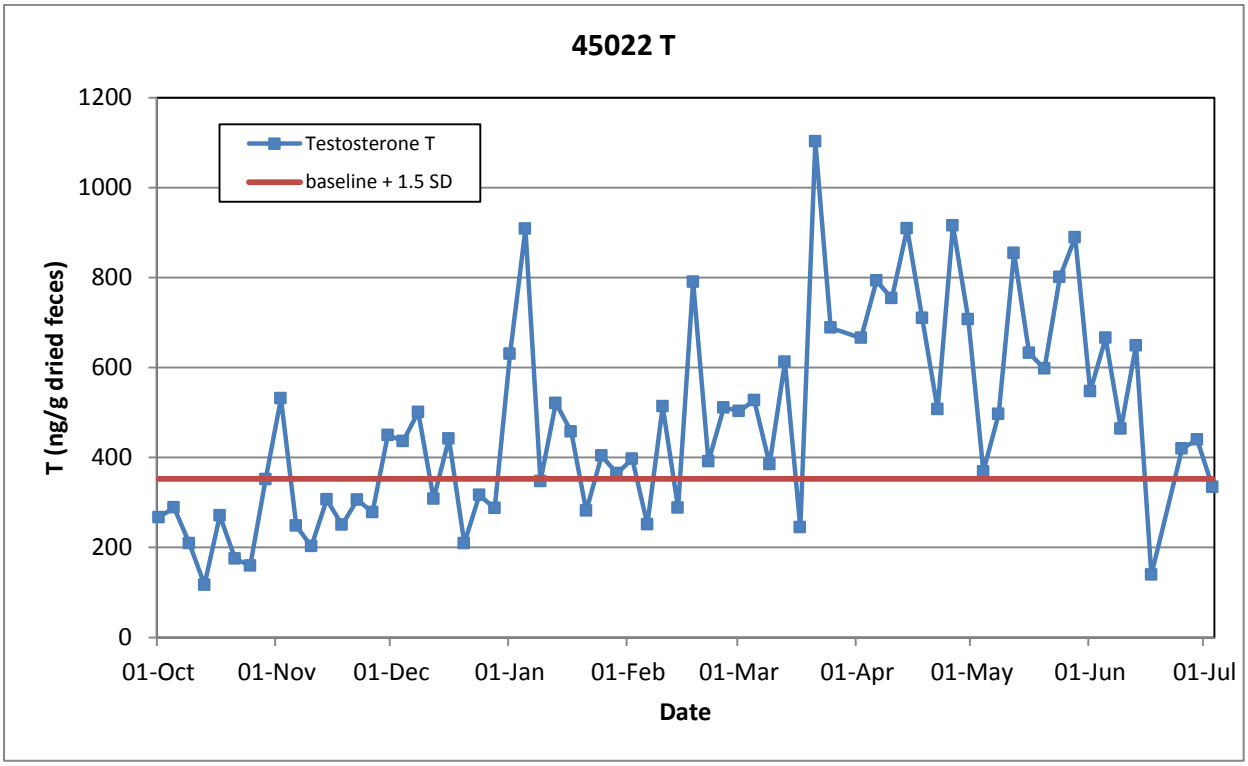

45022
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and P (ng/g dried feces)
P (ng/g dried feces)
2500
2000
1500
1000
500
0
12000
10000
8000
6000
4000
2000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

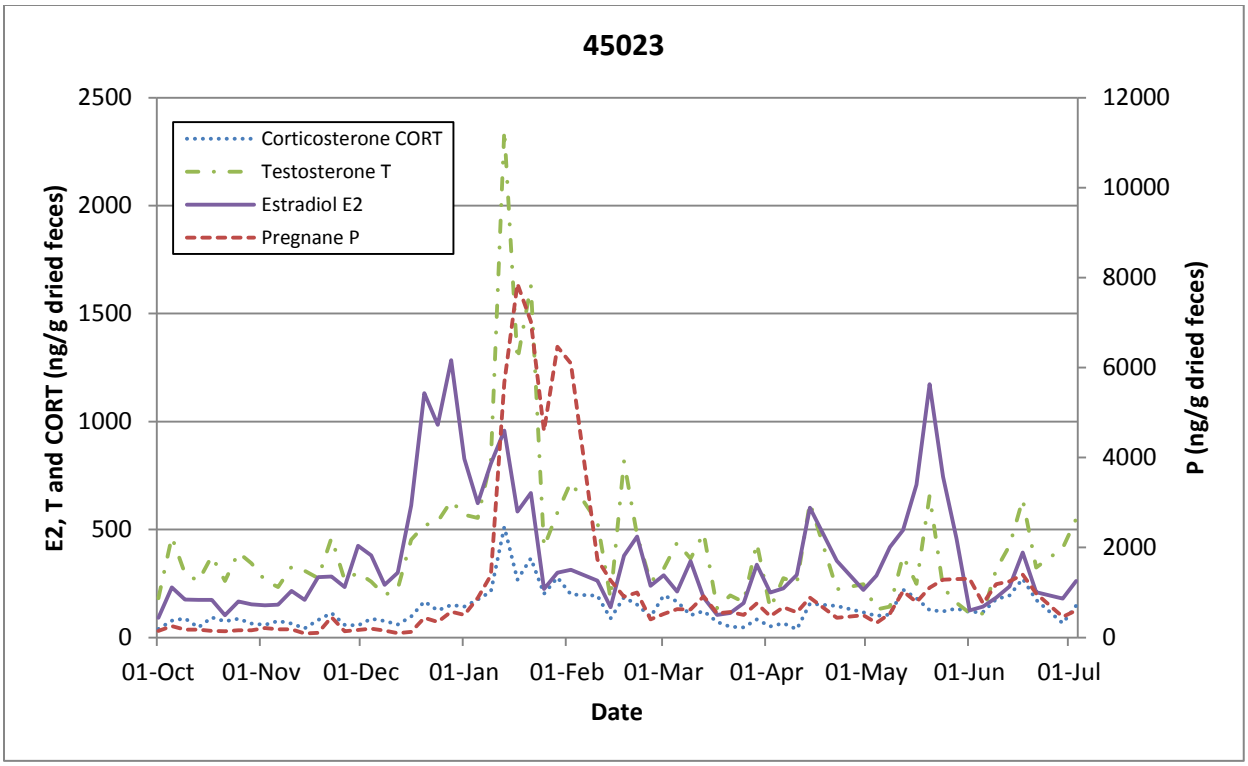

45022 Follicular changes
Length L
Width W
Surface Area SA
L, W (cm) & SA (cm²)
1.2
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

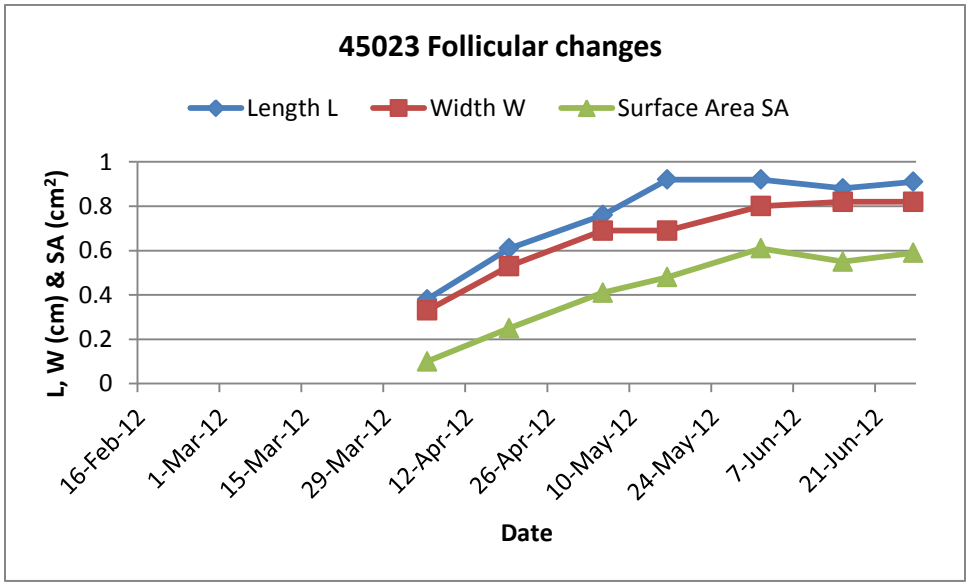

45022 CORT
Corticosterone CORT
baseline + 1.5 SD
CORT (ng/g dried feces)
300
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

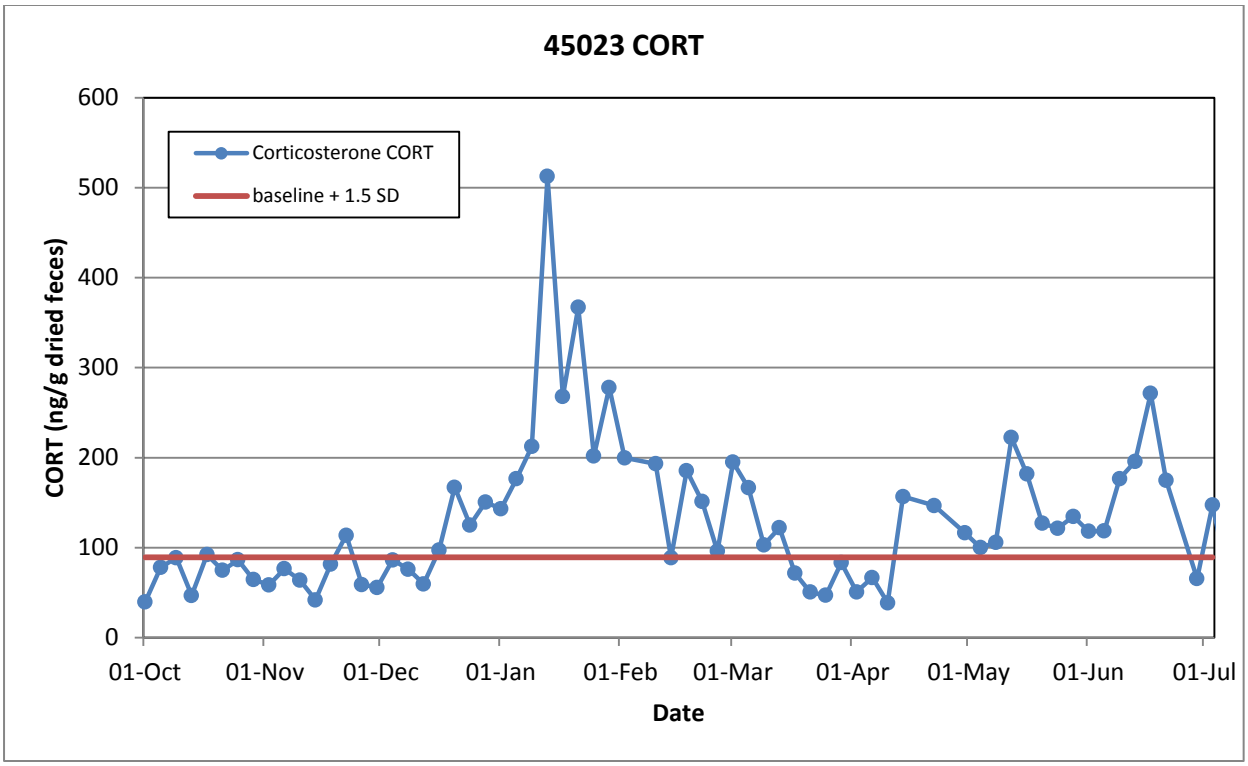

45022 P
Pregnane P
baseline + 1.5 SD
P (ng/g dried feces)
1600
1400
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

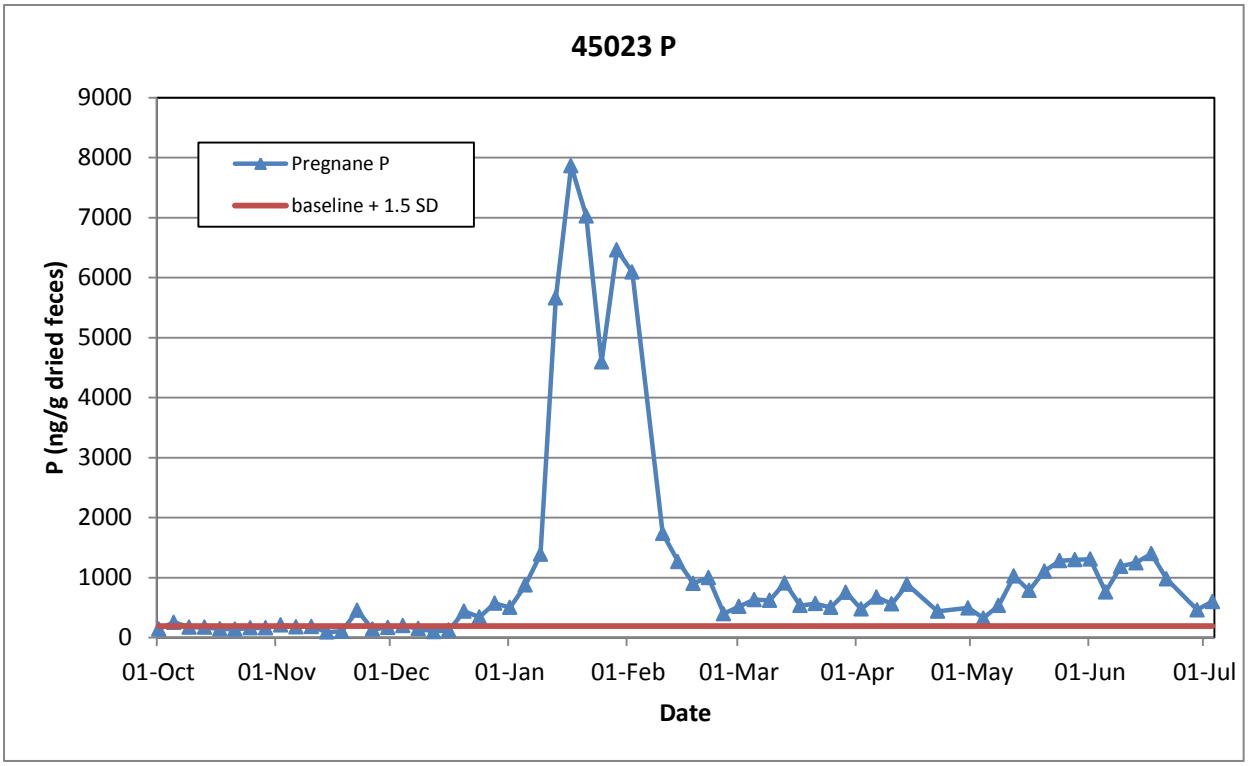

45022 E2
2500
2000
1500
1000
500
0
E2 (ng/g dried feces)
Estradiol E2
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

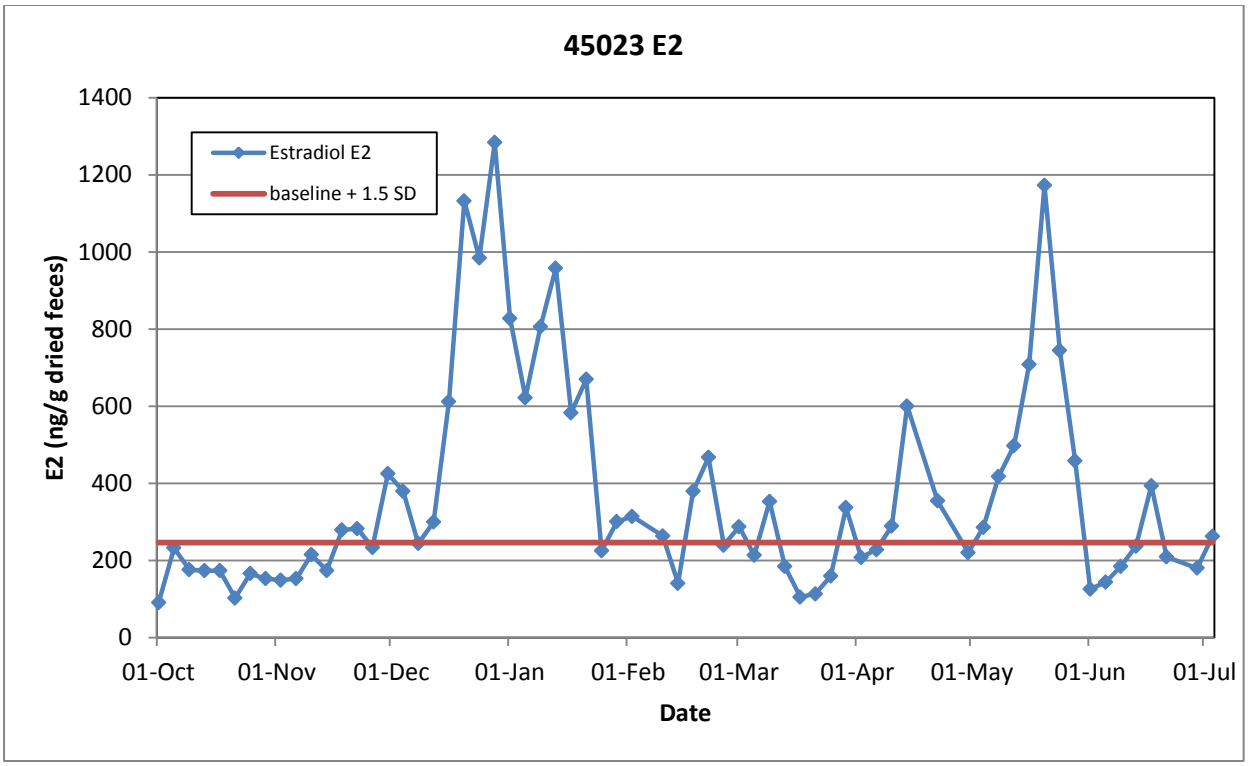

45022 T
1200
1000
800
600
400
200
0
T (ng/g dried feces)
Testosterone T
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

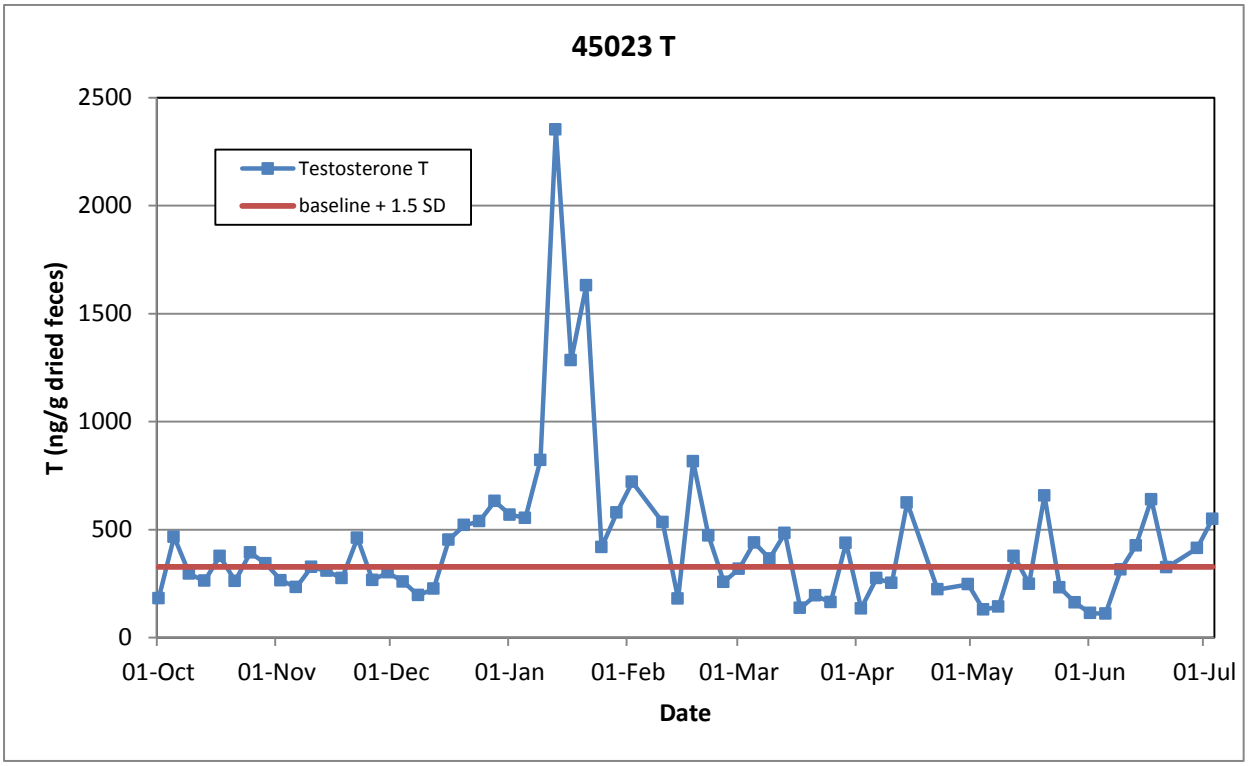

45023
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
2500
2000
1500
1000
500
0
12000
10000
8000
6000
4000
2000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

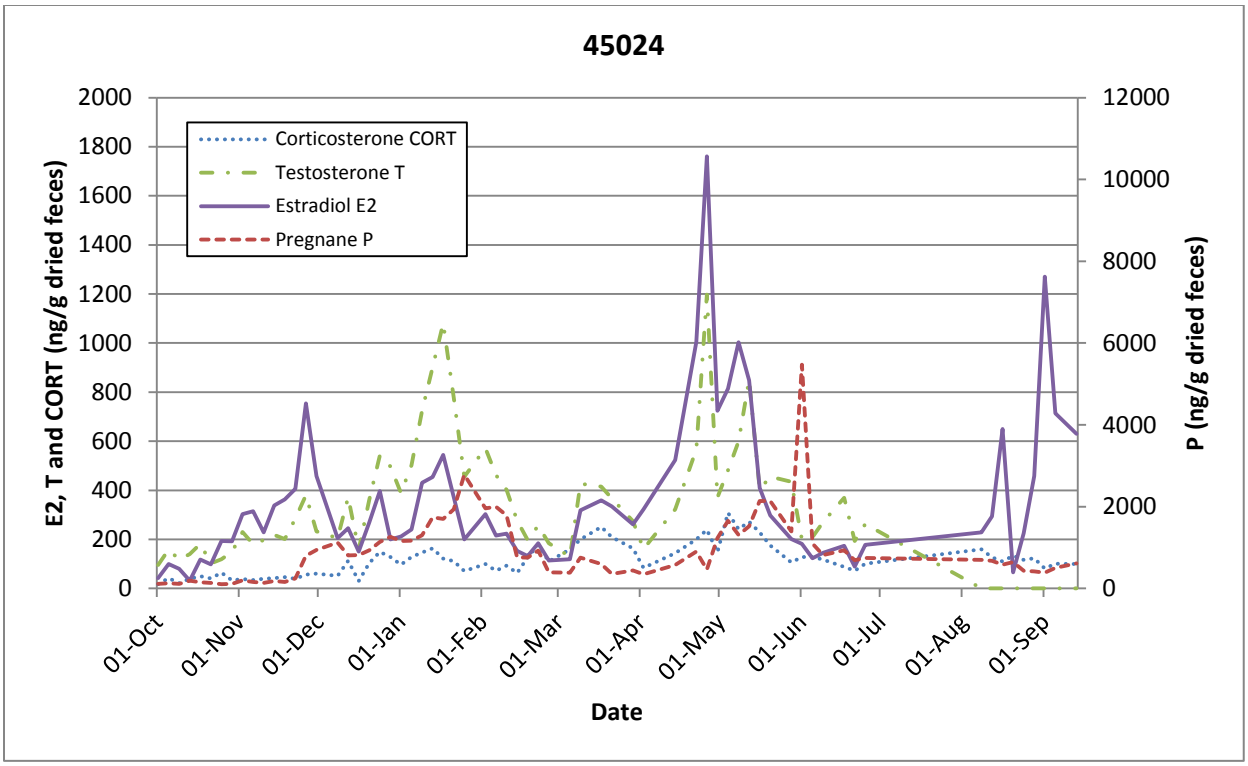

45023 Follicular changes
Length L
Width W
Surface Area SA
L, W (cm) & SA (cm²)
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

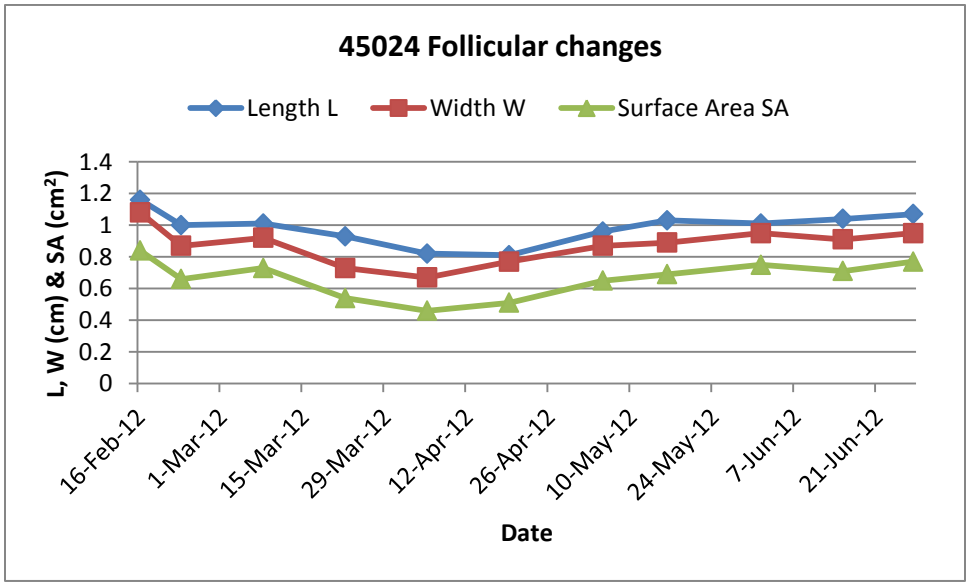

45023 CORT
Corticosterone CORT
baseline + 1.5 SD
CORT (ng/g dried feces)
600
500
400
300
200
100
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

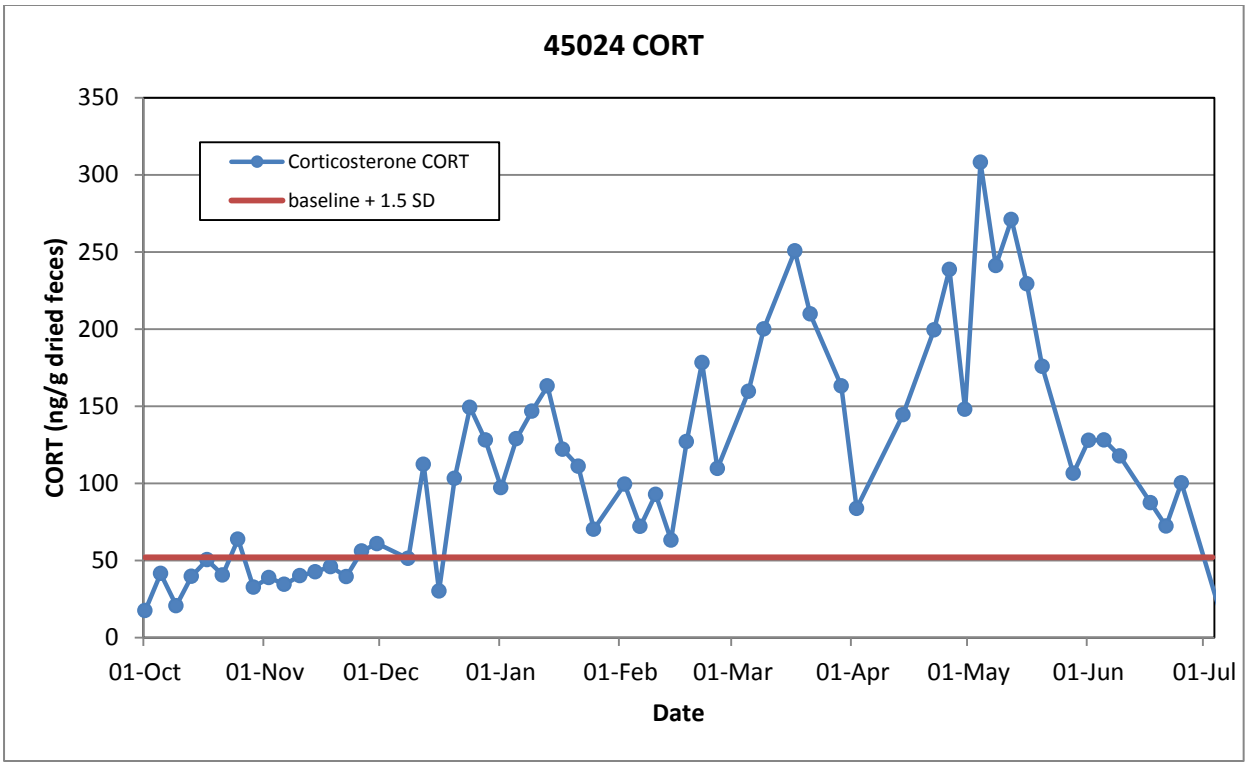

45023 P
Pregnane P
baseline + 1.5 SD
P (ng/g dried feces)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

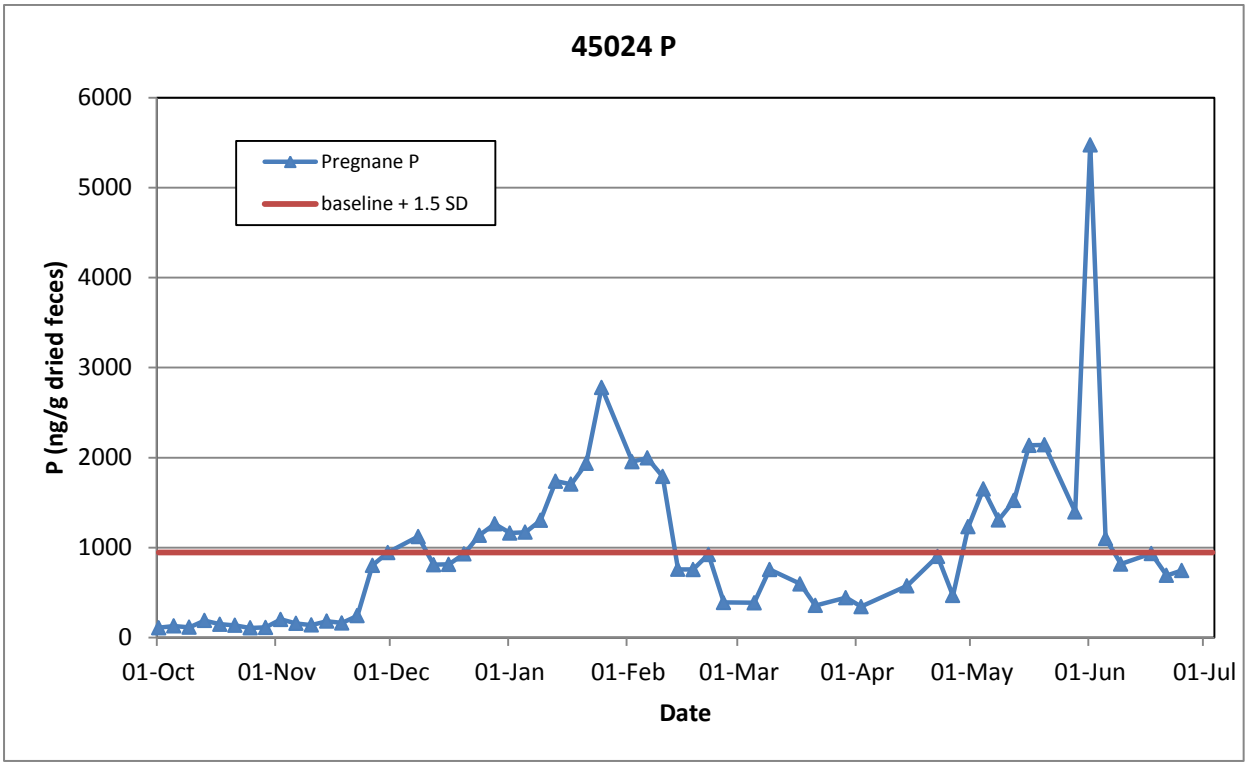

45023 E2
Estradiol E2
baseline + 1.5 SD
E2 (ng/g dried feces)
1400
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

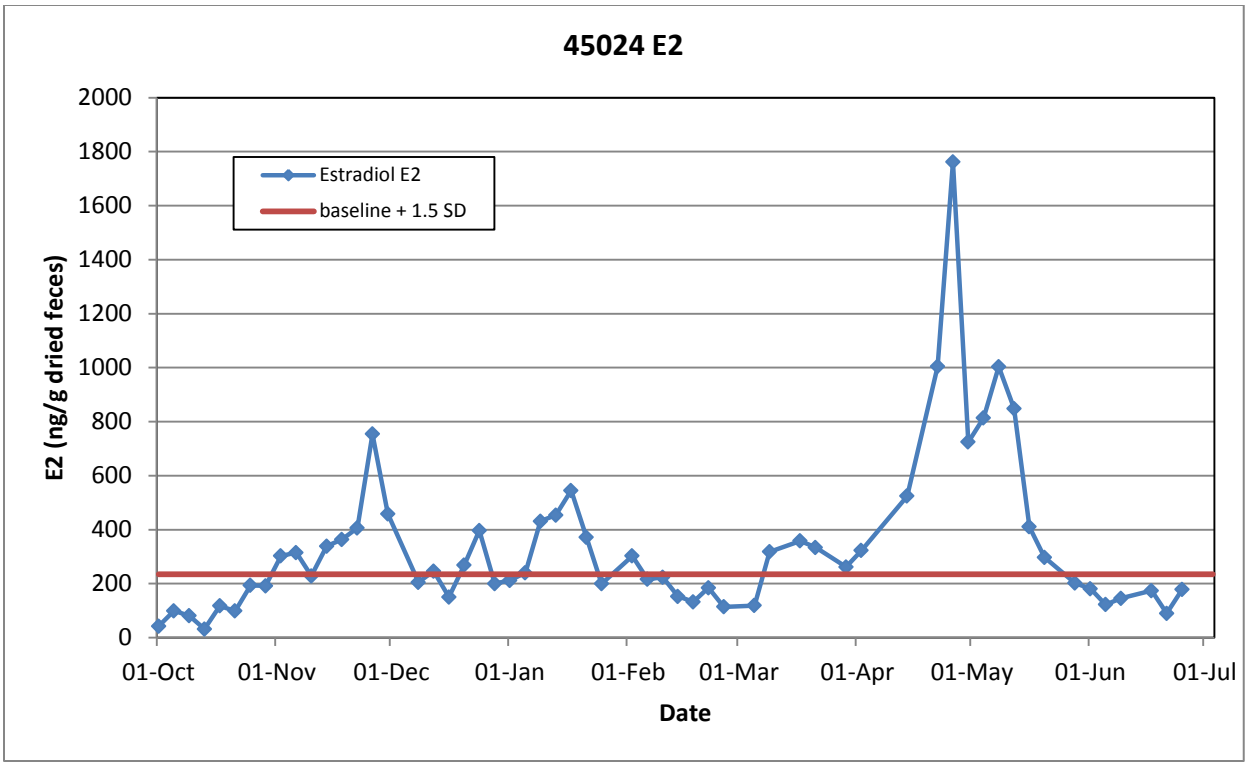

45023 T
Testosterone T
baseline + 1.5 SD
T (ng/g dried feces)
2500
2000
1500
1000
500
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

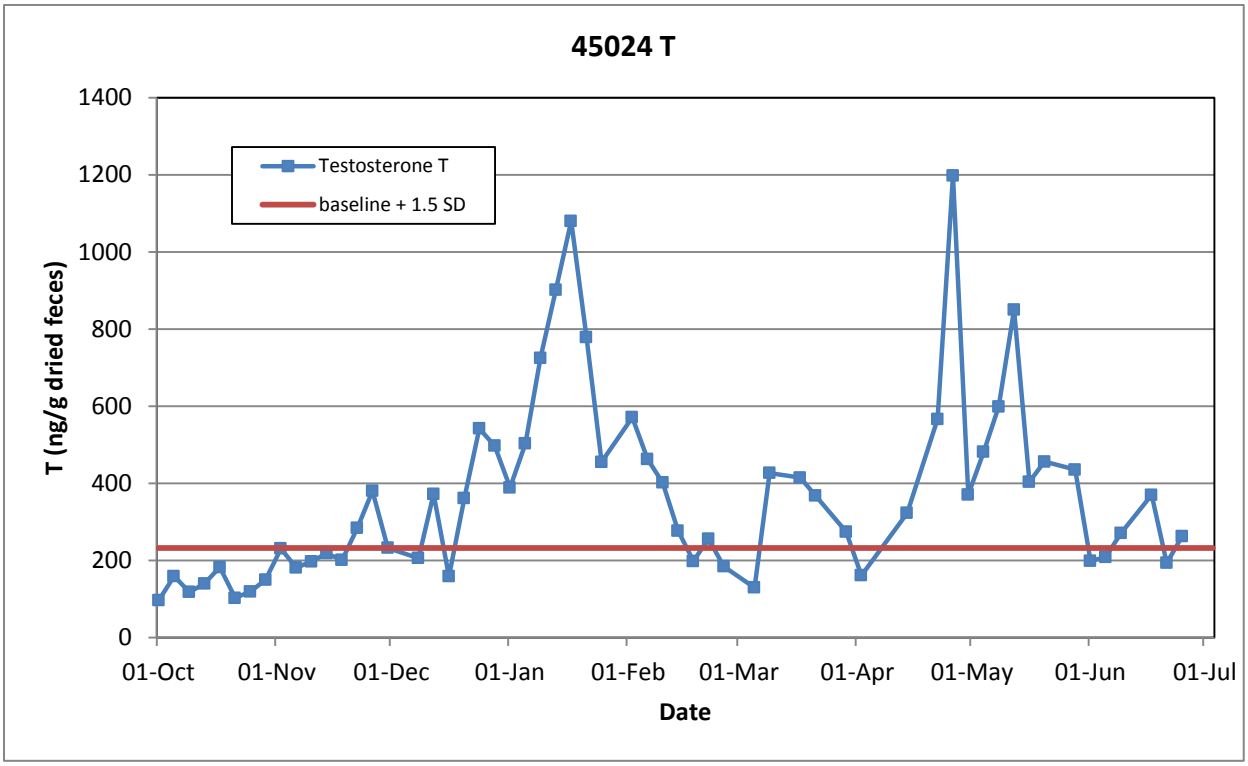

45024
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
12000
10000
8000
6000
4000
2000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
01-Aug
01-Sep
Date

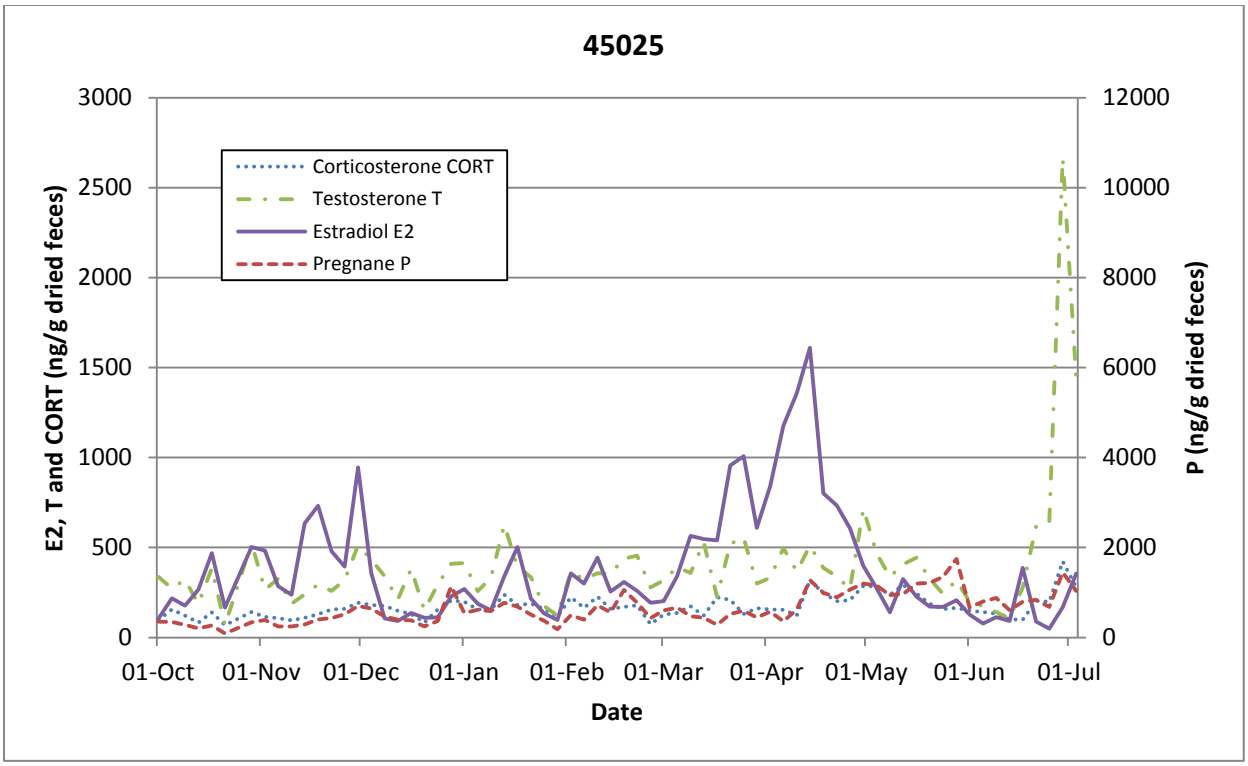

45024 Follicular changes
Length L
Width W
Surface Area SA
L, W (cm) & SA (cm²)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

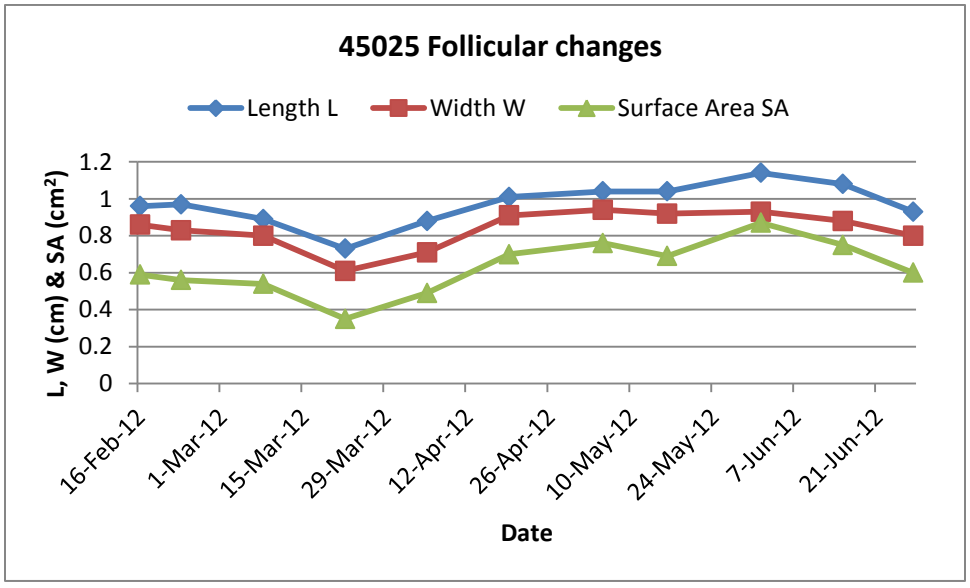

45024 CORT
Corticosterone CORT
baseline + 1.5 SD
CORT (ng/g dried feces)
350
300
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

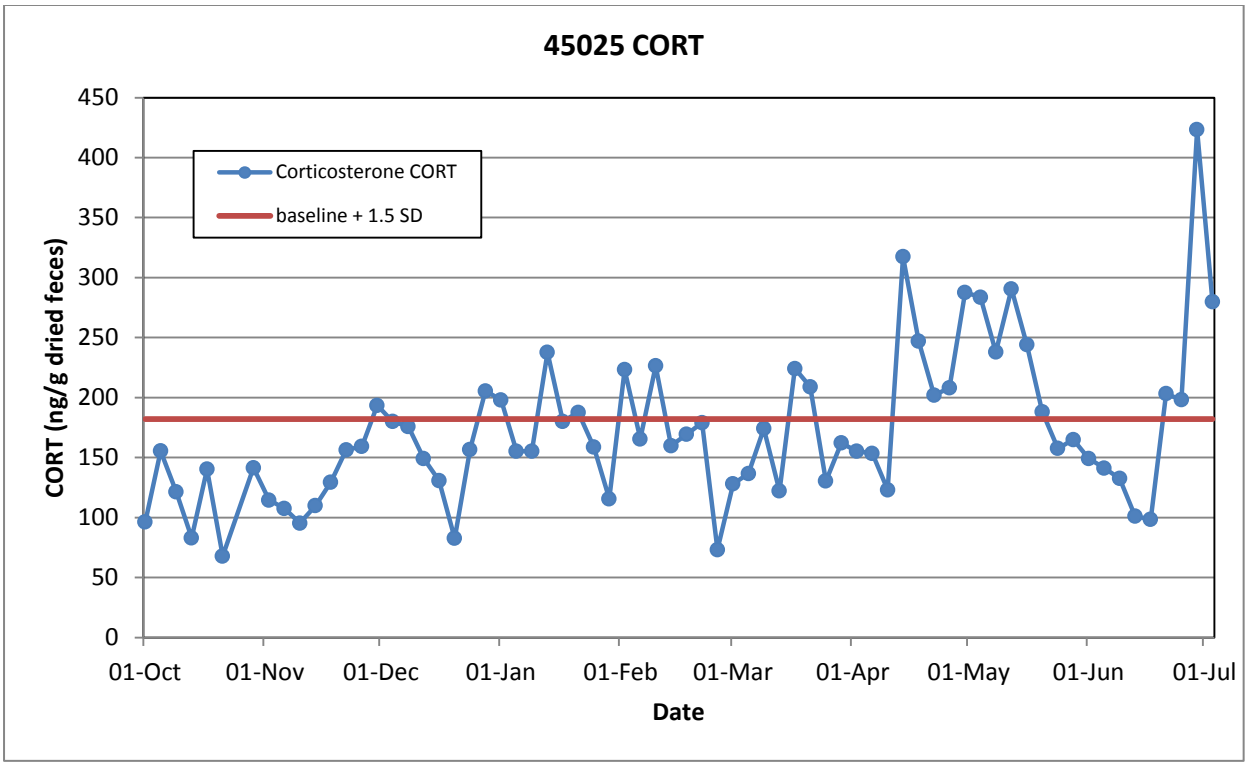

45024 P
Pregnane P
baseline + 1.5 SD
P (ng/g dried feces)
6000
5000
4000
3000
2000
1000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

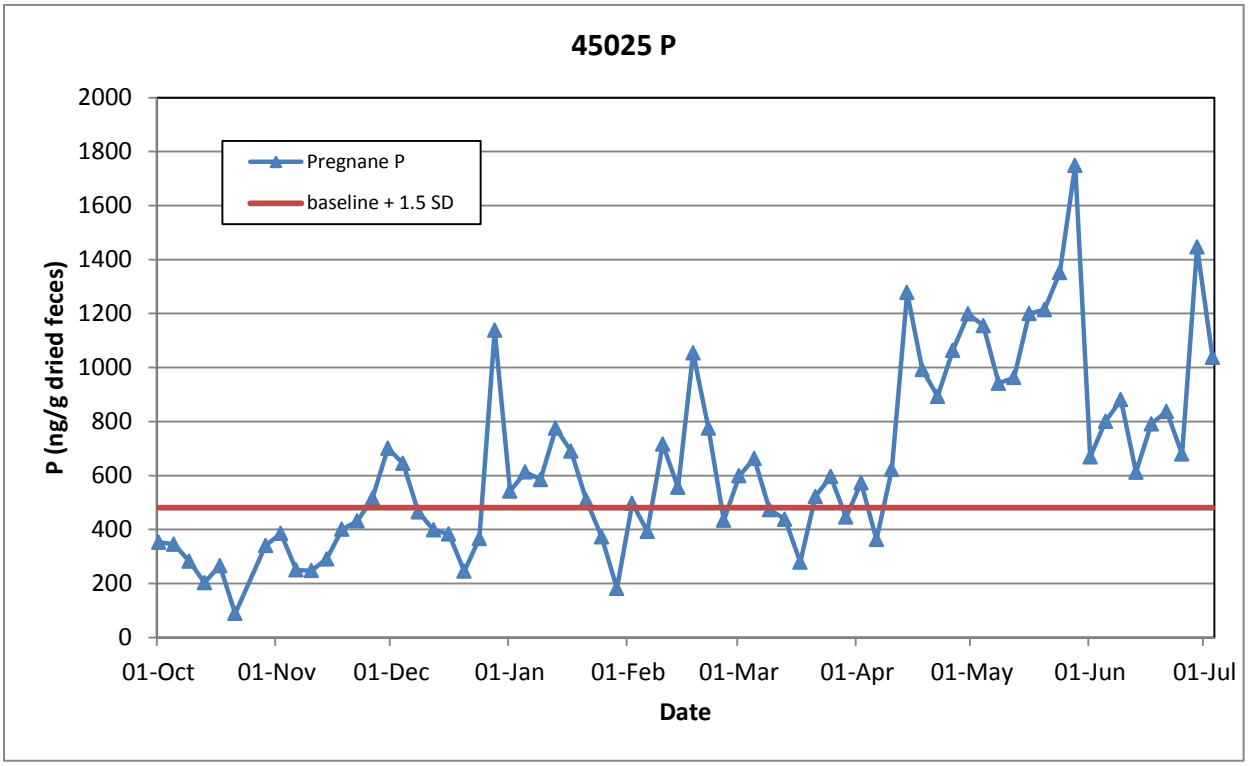

45024 E2
2000
1800
1600
1400
1200
1000
800
600
400
200
0
E2 (ng/g dried feces)
Estradiol E2
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

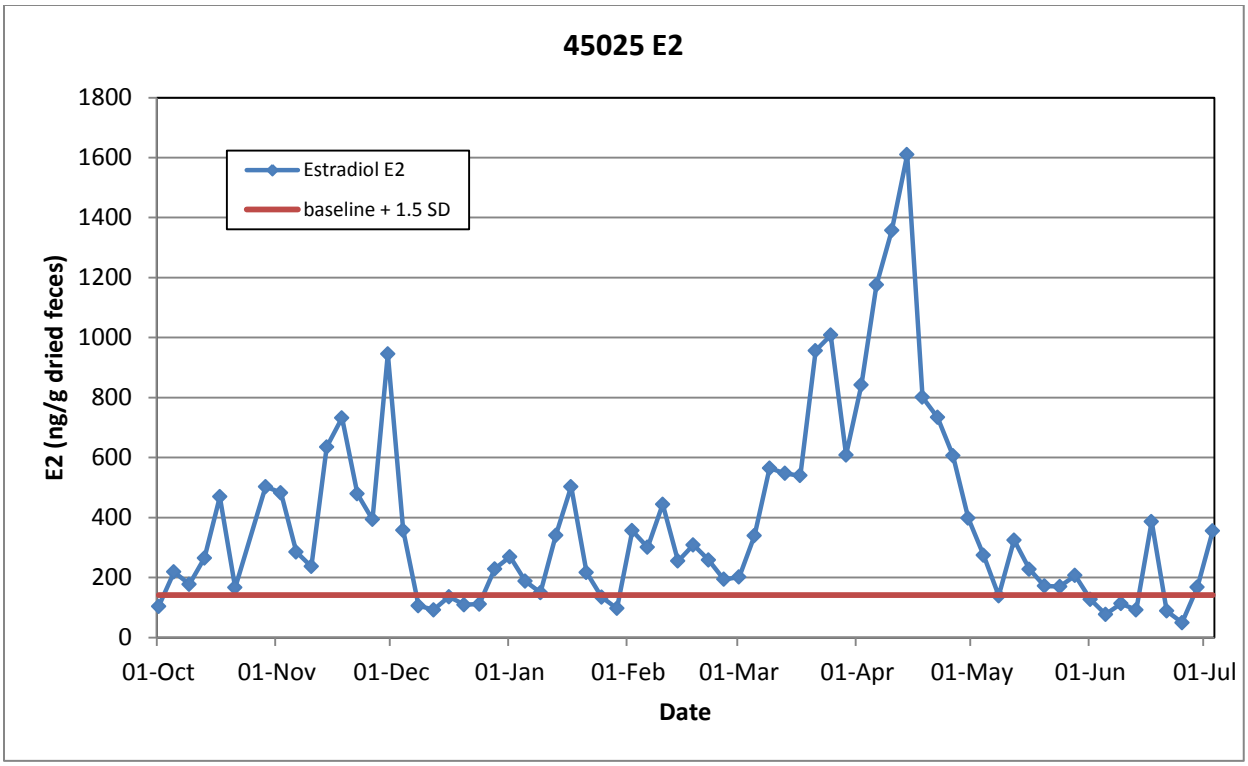

45024 T
1400
1200
1000
800
600
400
200
0
T (ng/g dried feces)
Testosterone T
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

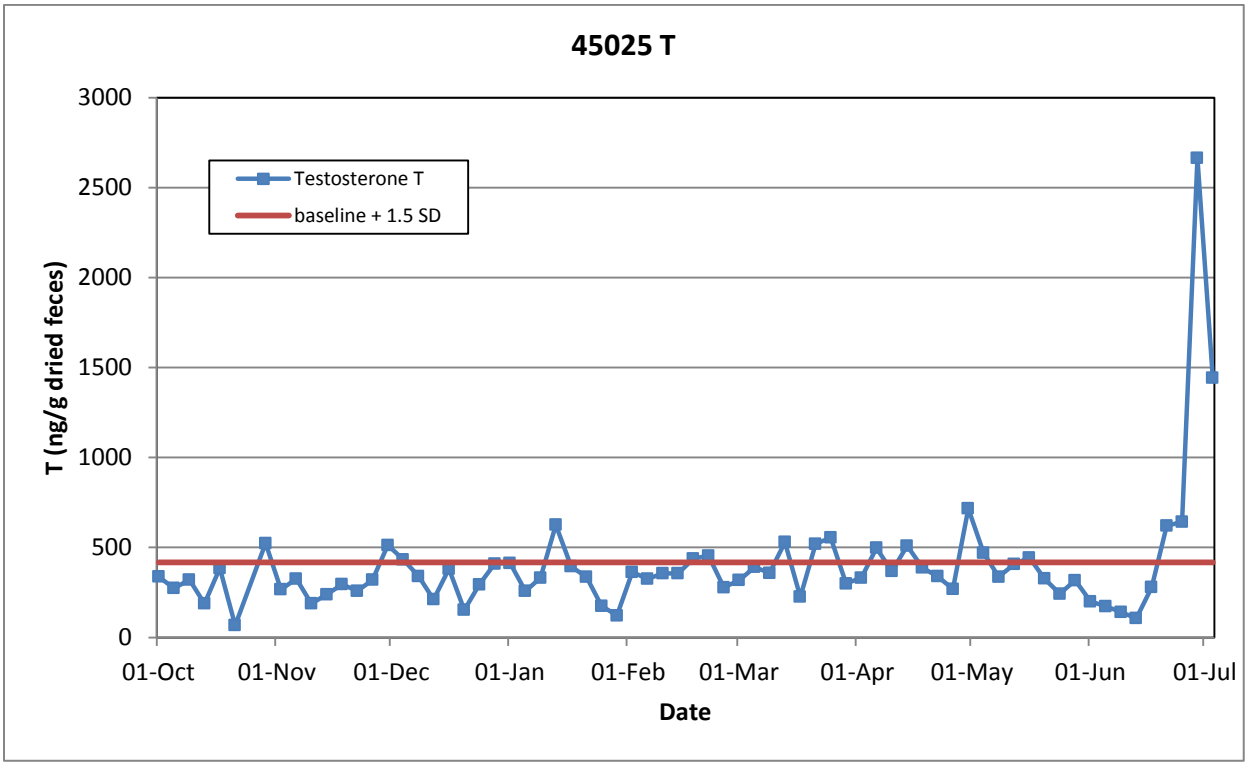

45025
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
3000
2500
2000
1500
1000
500
0
12000
10000
8000
6000
4000
2000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

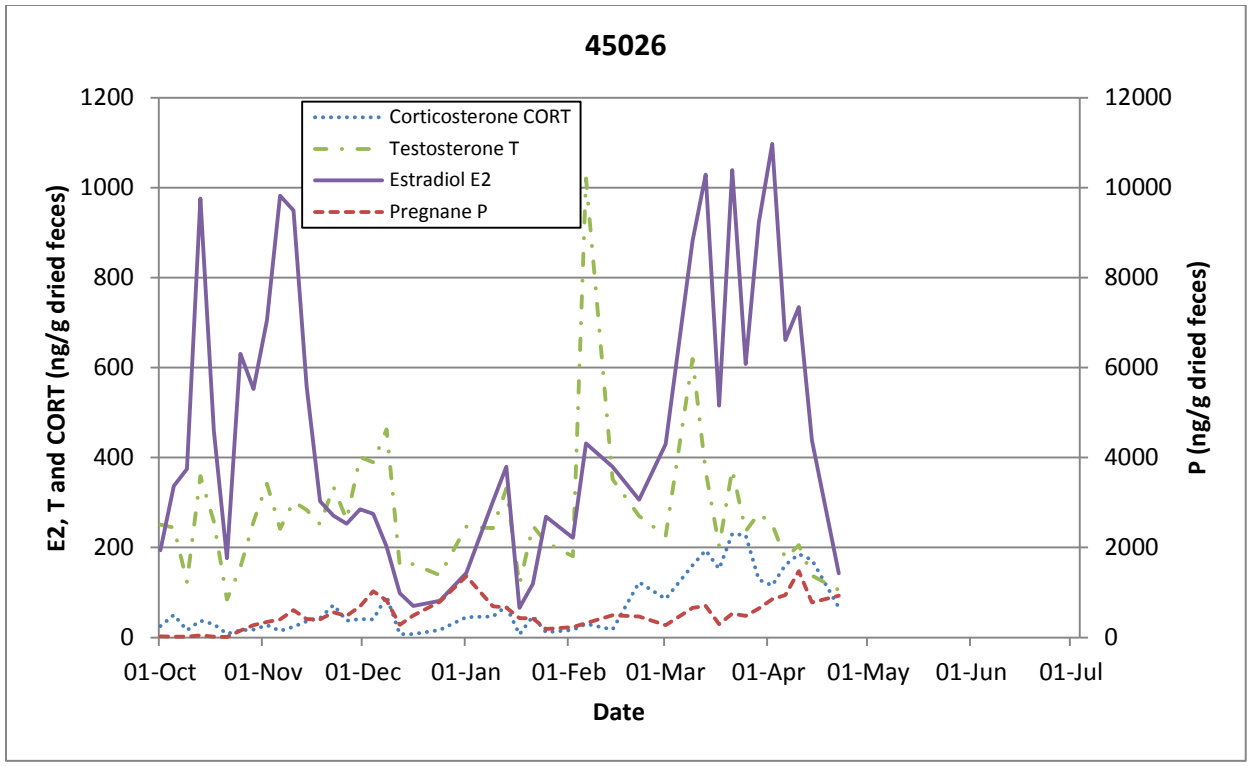

45025 Follicular changes
Length L
Width W
Surface Area SA
L, W (cm) & SA (cm²)
1.2
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

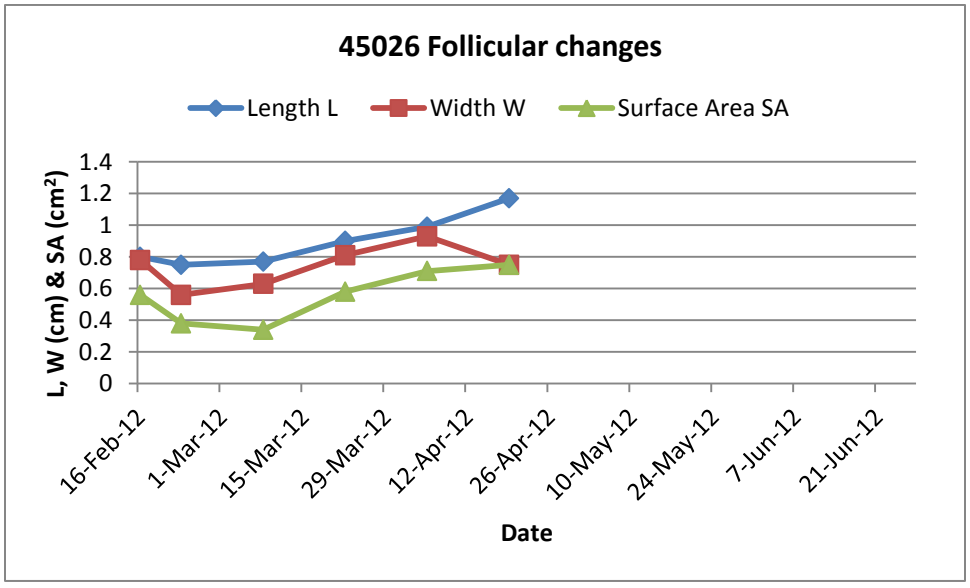

45025 CORT
Corticosterone CORT
baseline + 1.5 SD
CORT (ng/g dried feces)
450
400
350
300
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

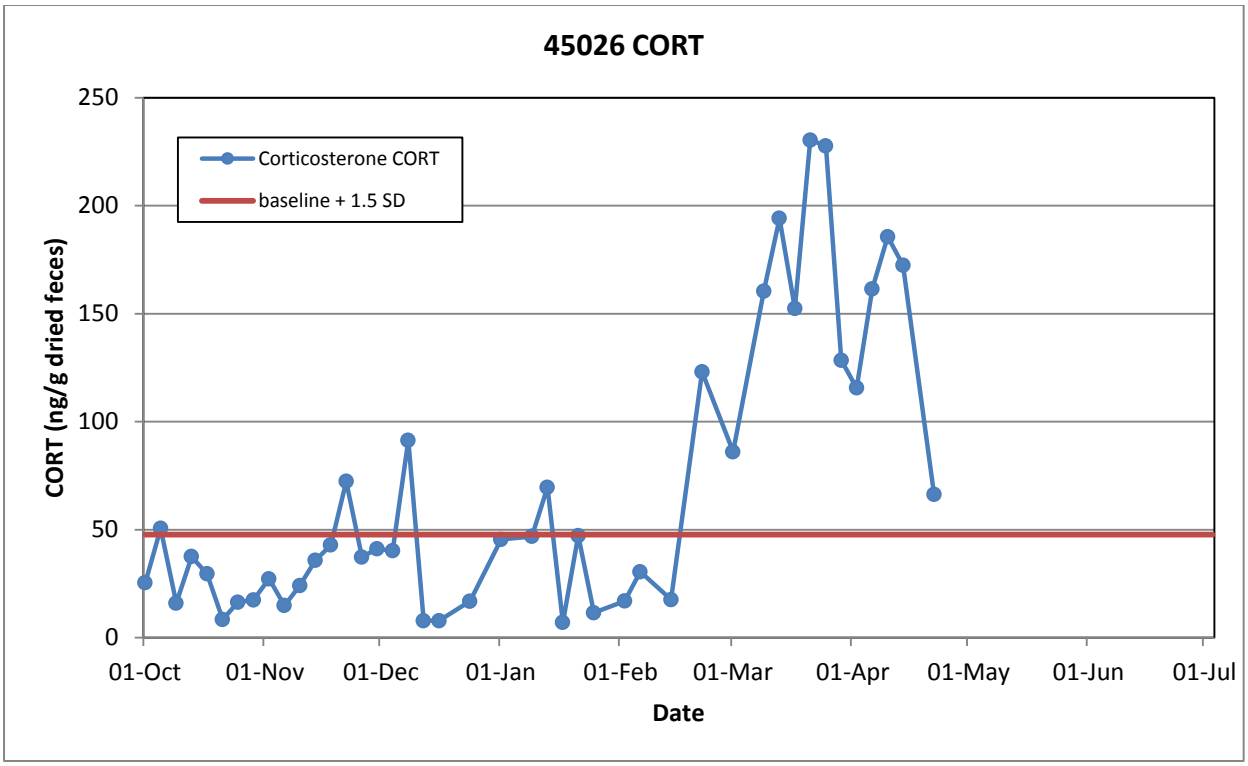

45025 P
Pregnane P
baseline + 1.5 SD
P (ng/g dried feces)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

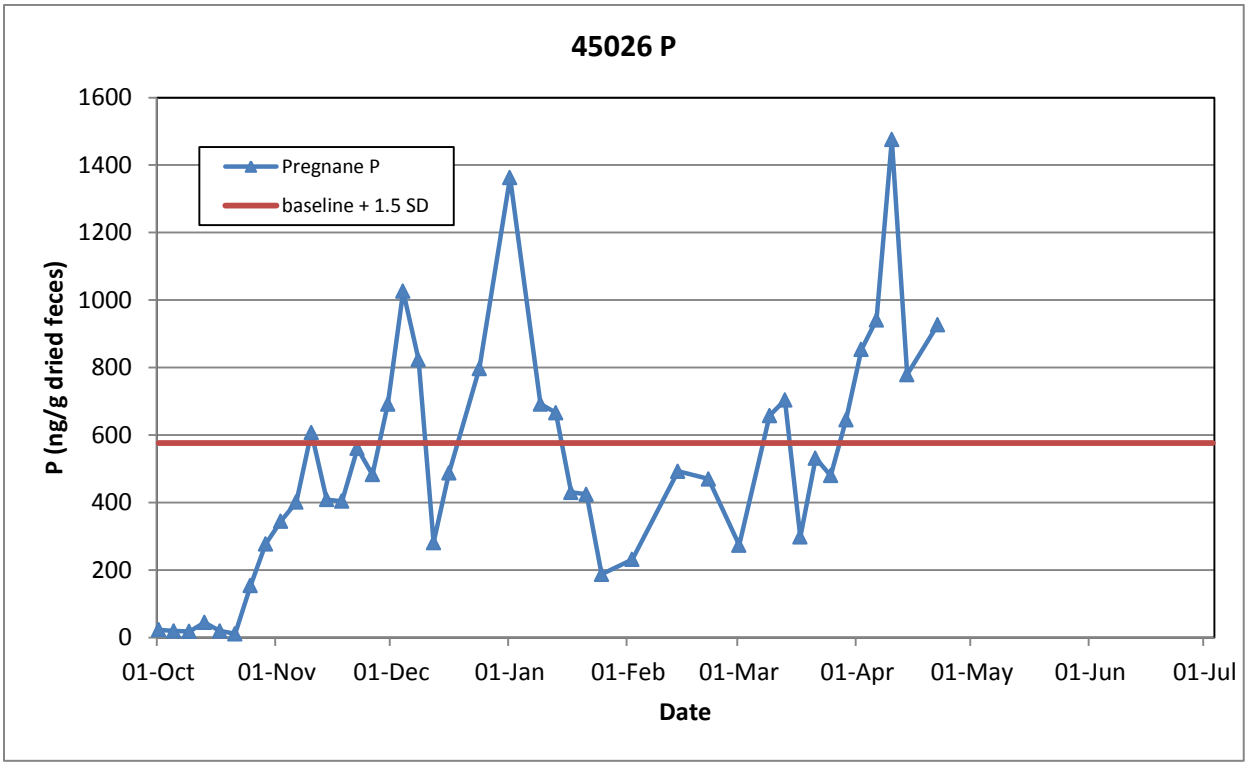

45025 E2
1800
1600
1400
1200
1000
800
600
400
200
0
E2 (ng/g dried feces)
Estradiol E2
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

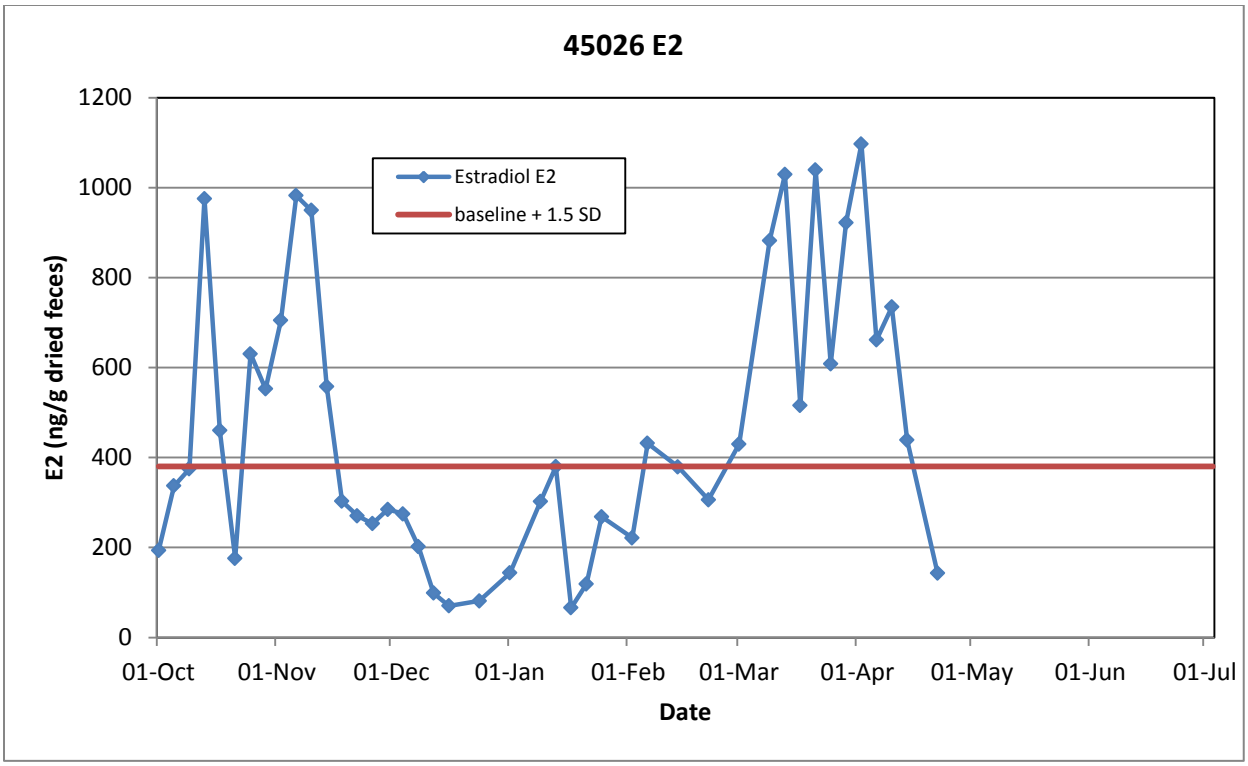

45025 T
3000
2500
2000
1500
1000
500
0
T (ng/g dried feces)
Testosterone T
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

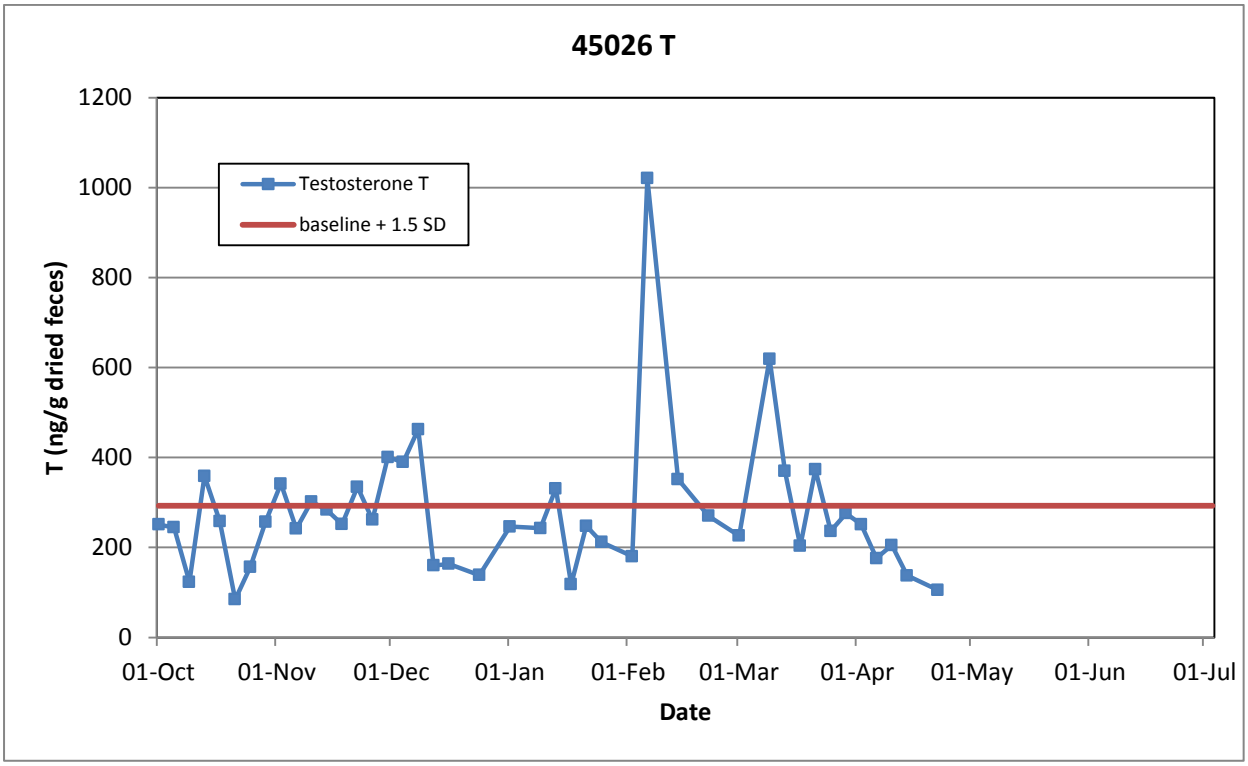

45026
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
1200
1000
800
600
400
200
0
12000
10000
8000
6000
4000
2000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

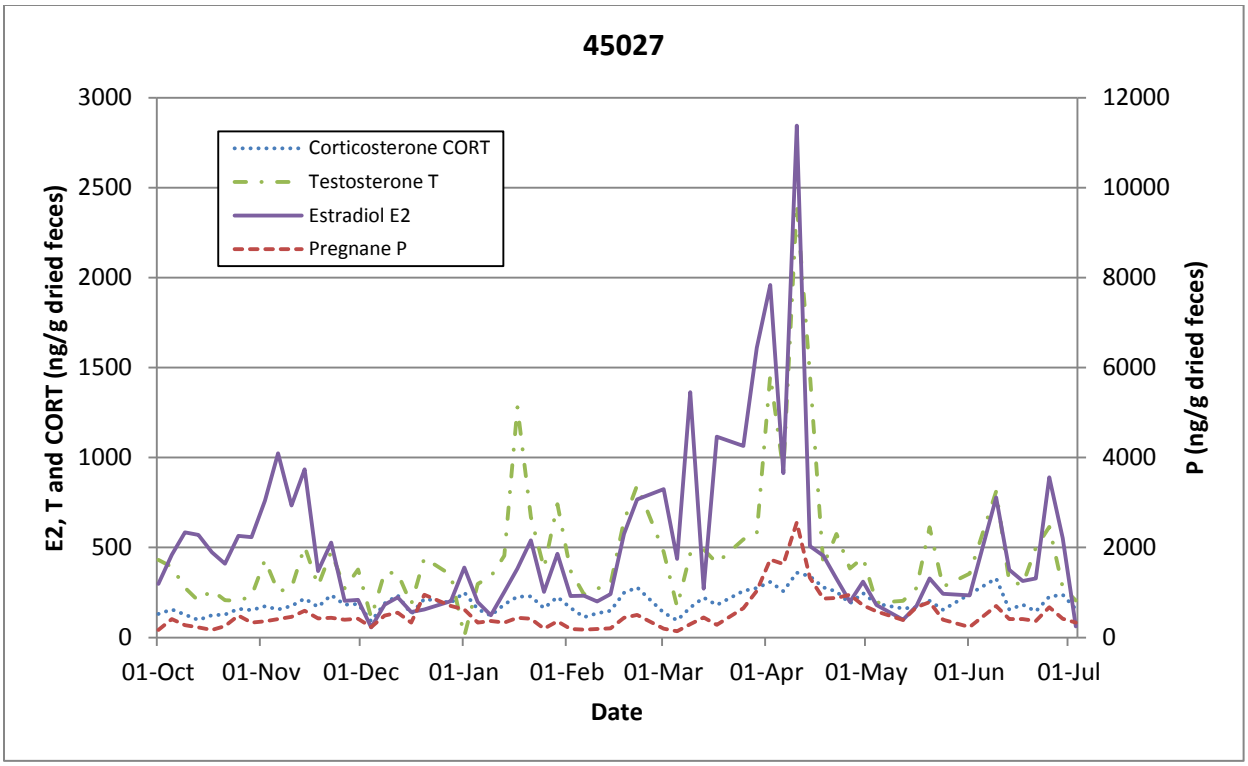

45026 Follicular changes
Length L
Width W
Surface Area SA
L, W (cm) & SA (cm²)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

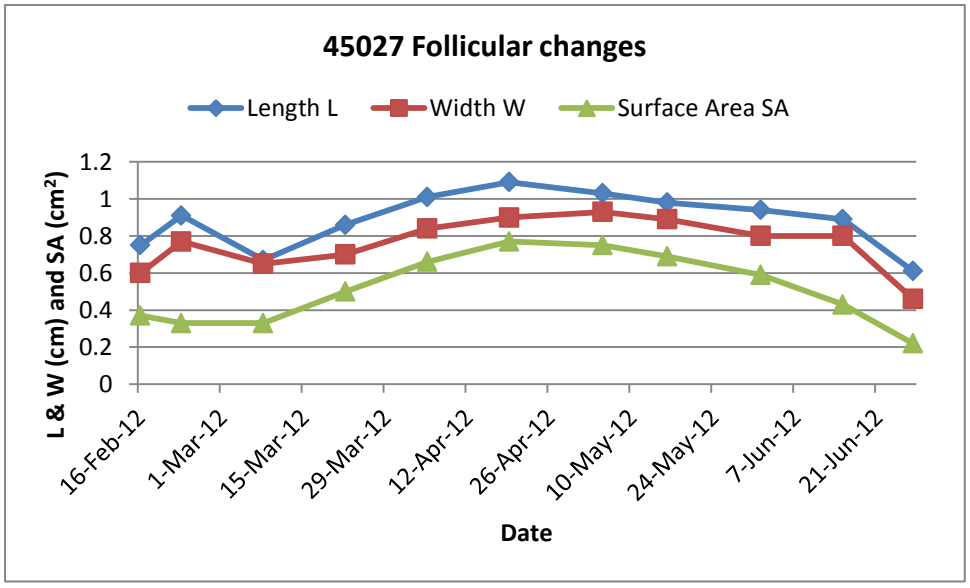

45026 CORT
Corticosterone CORT
baseline + 1.5 SD
CORT (ng/g dried feces)
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

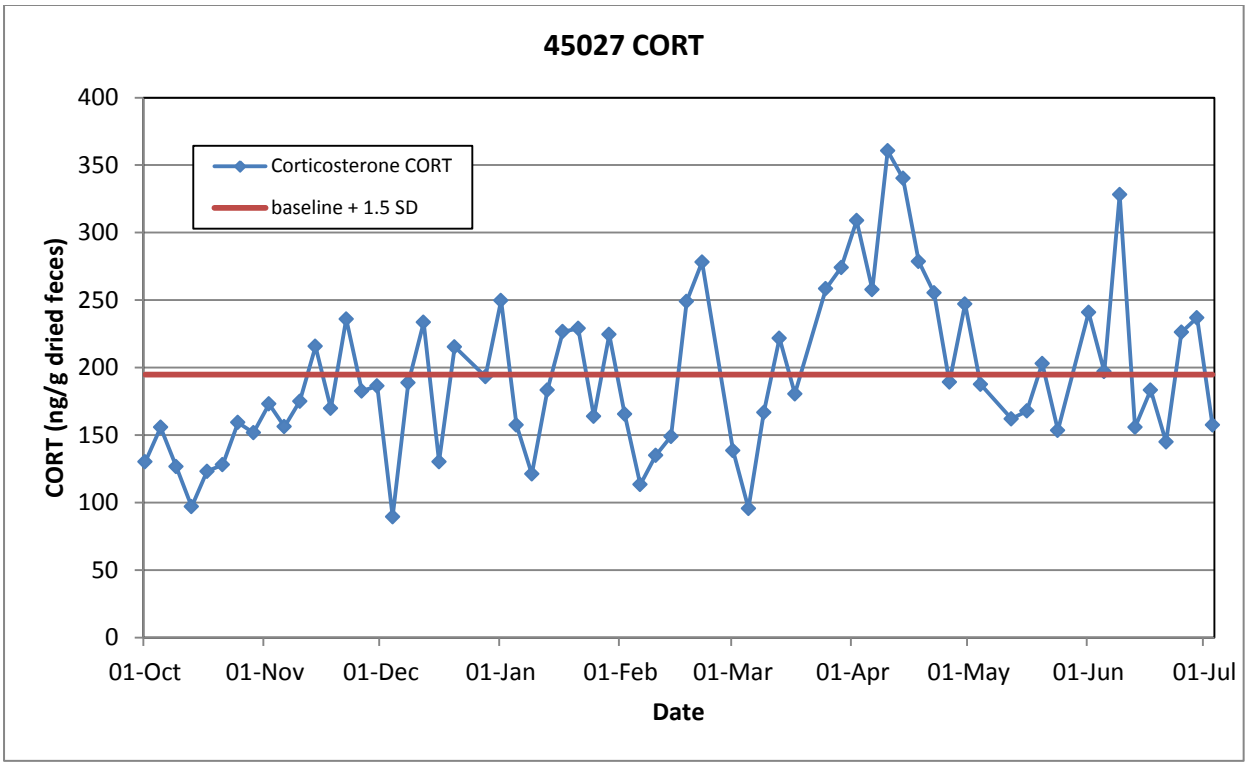

45026 P
Pregnane P
baseline + 1.5 SD
P (ng/g dried feces)
1600
1400
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

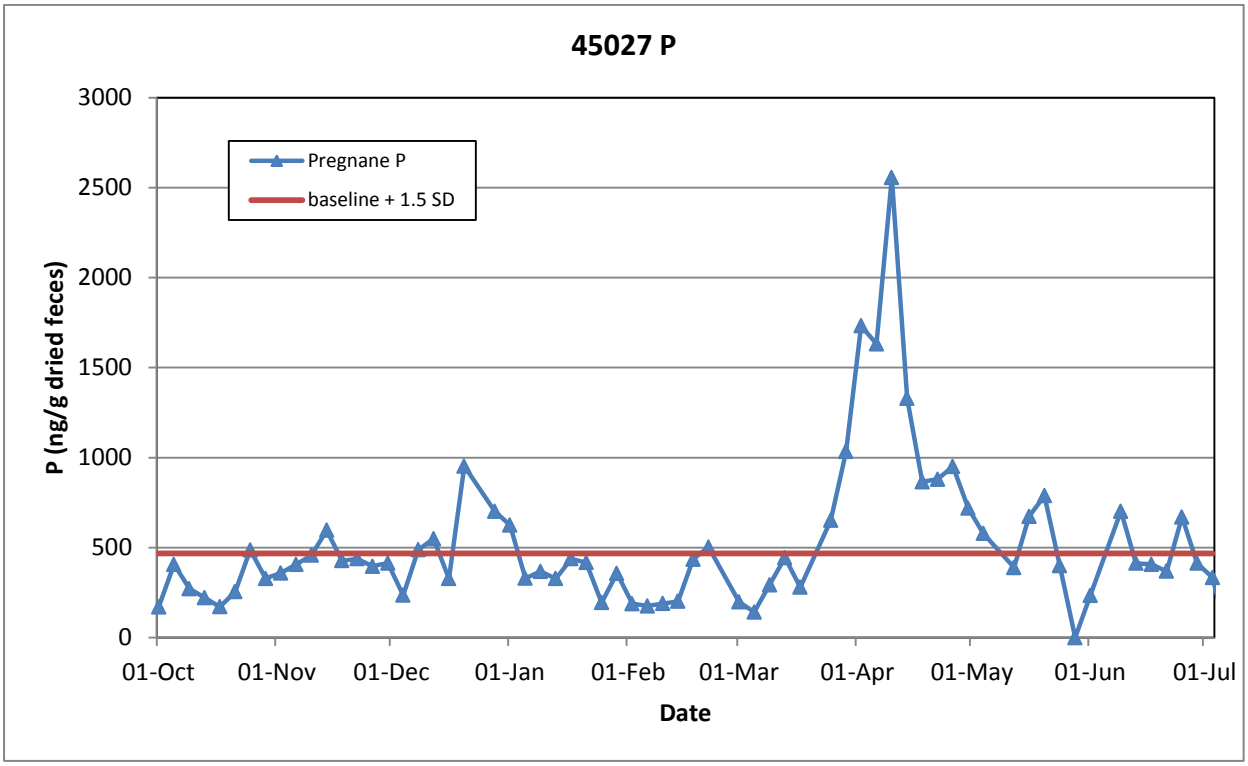

45026 E2
1200
1000
800
600
400
200
0
E2 (ng/g dried feces)
Estradiol E2
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

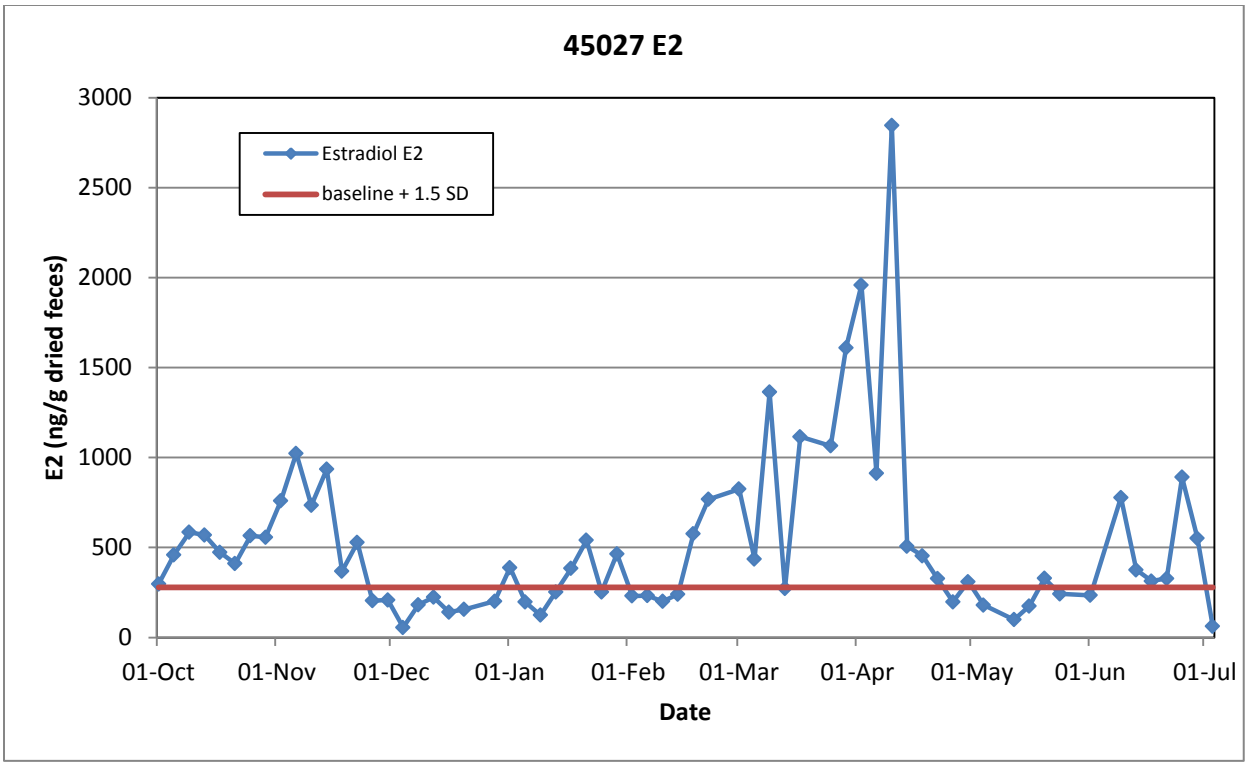

45026 T
1200
1000
800
600
400
200
0
T (ng/g dried feces)
Testosterone T
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

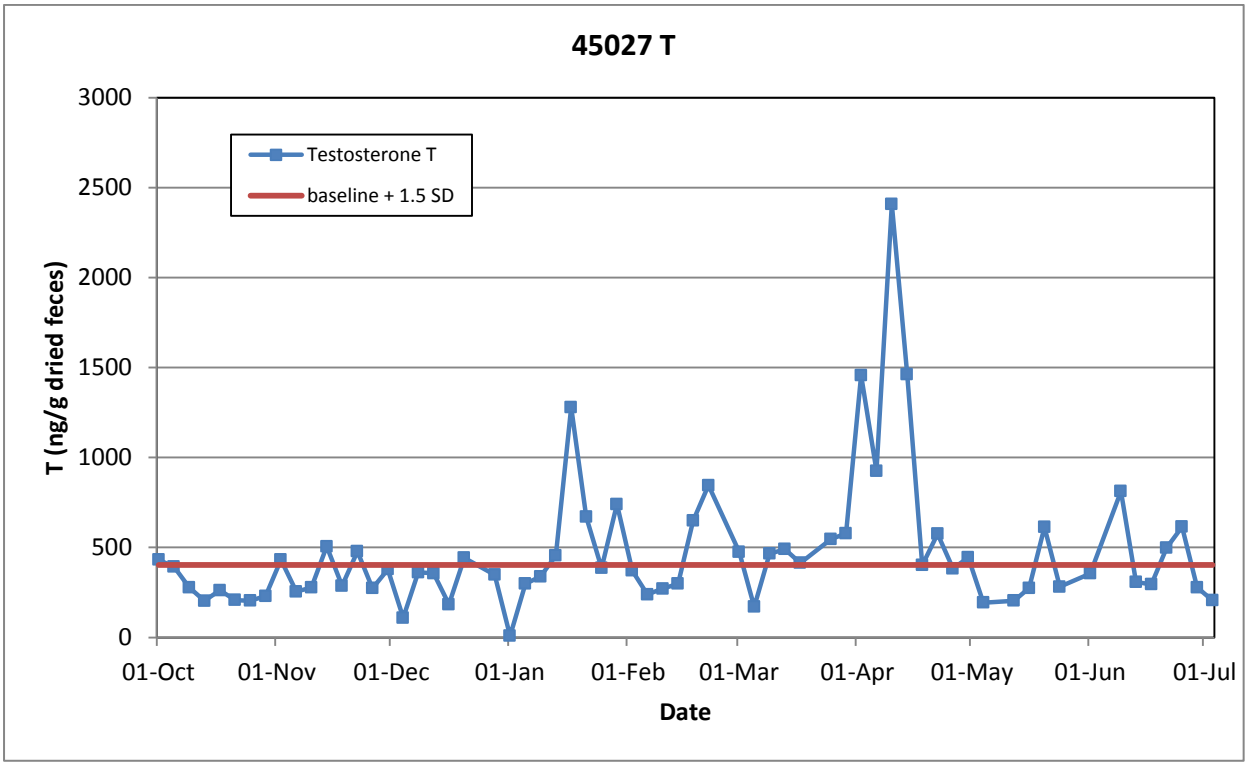

45027
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
3000
2500
2000
1500
1000
500
0
12000
10000
8000
6000
4000
2000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

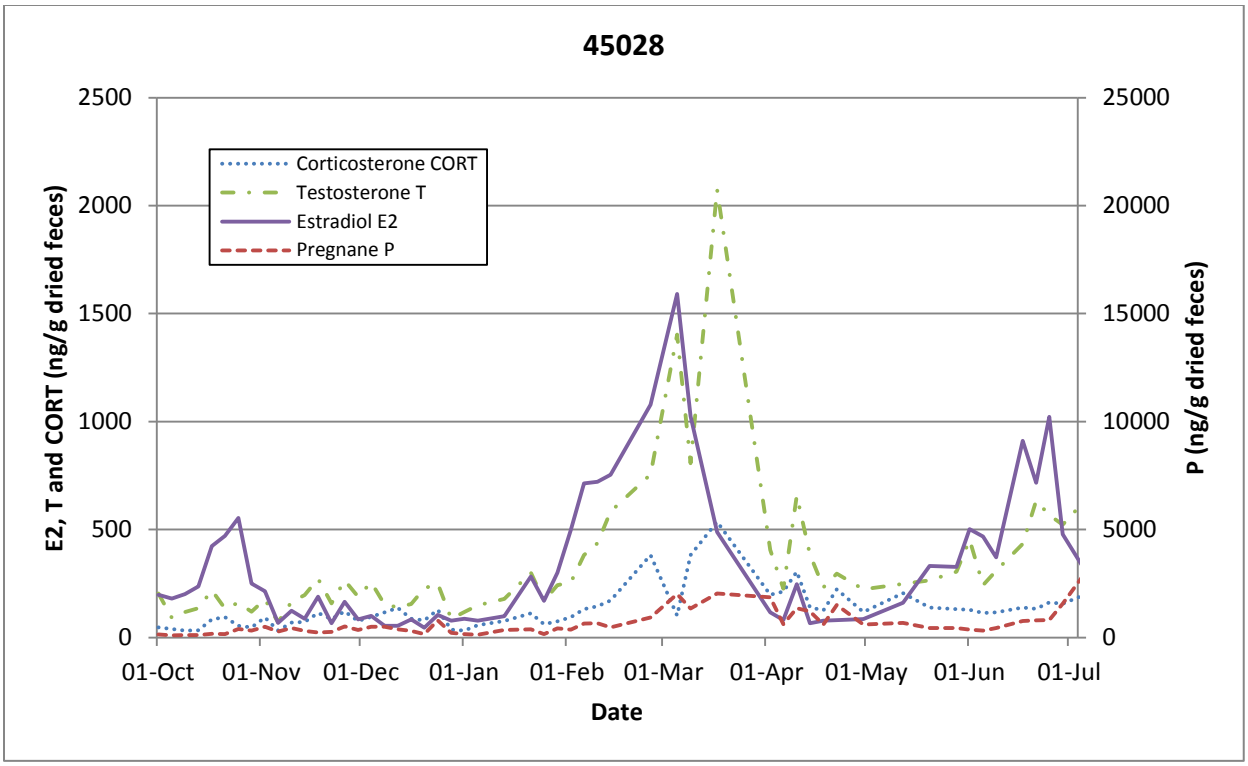

45027 Follicular changes
Length L
Width W
Surface Area SA
L & W (cm) and SA (cm²)
1.2
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

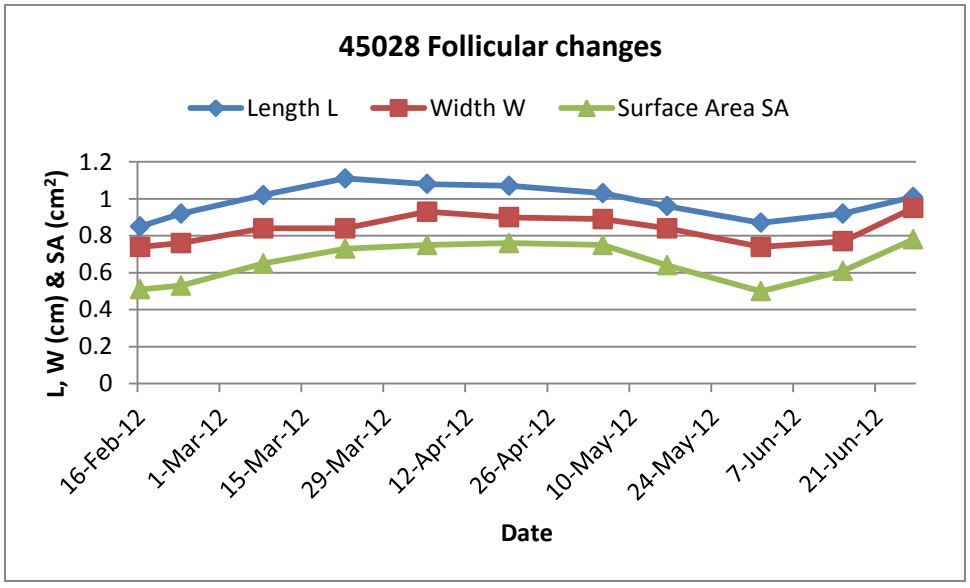

45027 CORT
Corticosterone CORT
baseline + 1.5 SD
CORT (ng/g dried feces)
400
350
300
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

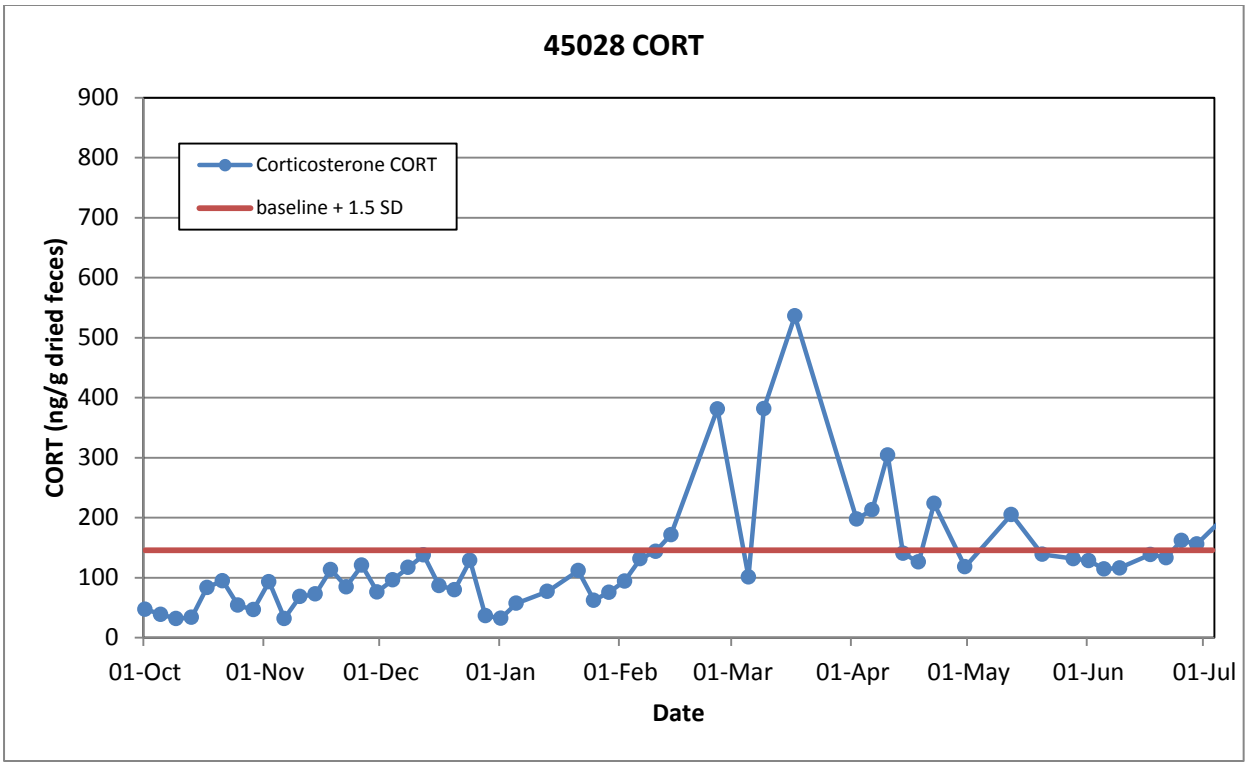

45027 P
Pregnane P
baseline + 1.5 SD
P (ng/g dried feces)
3000
2500
2000
1500
1000
500
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

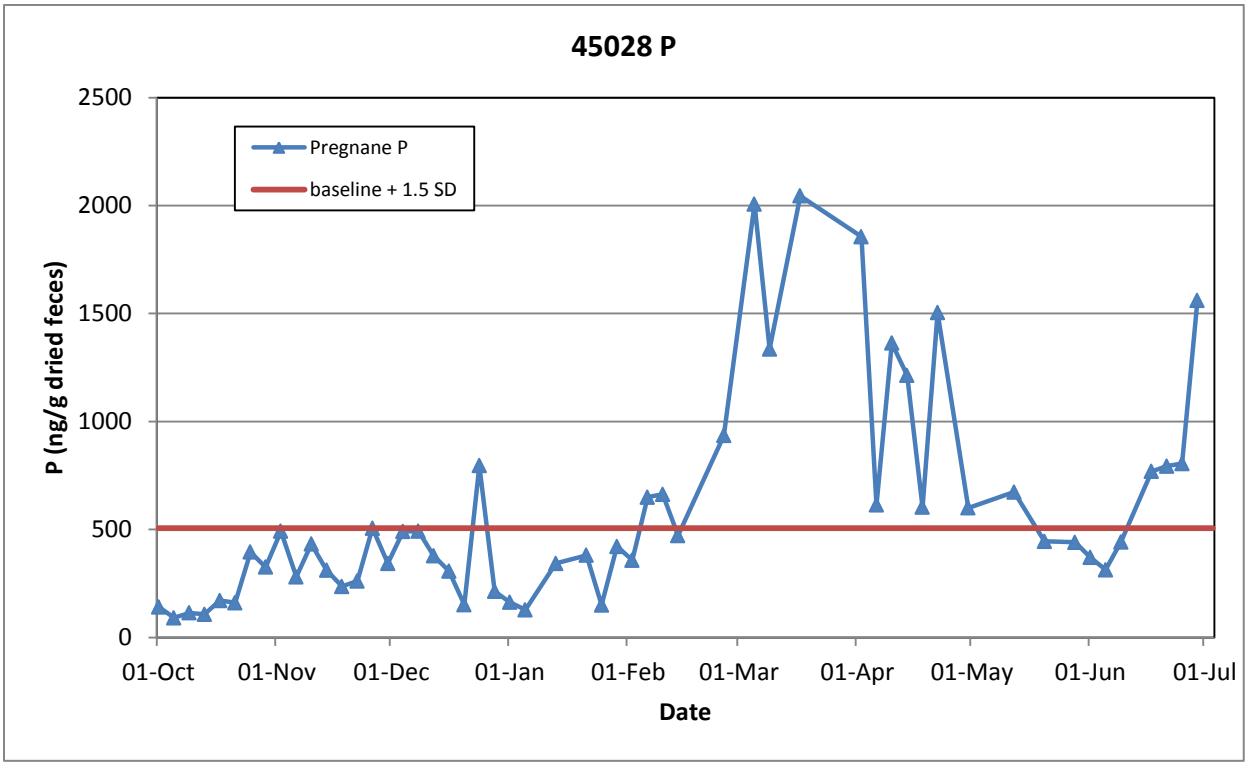

45027 E2
Estradiol E2
baseline + 1.5 SD
E2 (ng/g dried feces)
3000
2500
2000
1500
1000
500
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

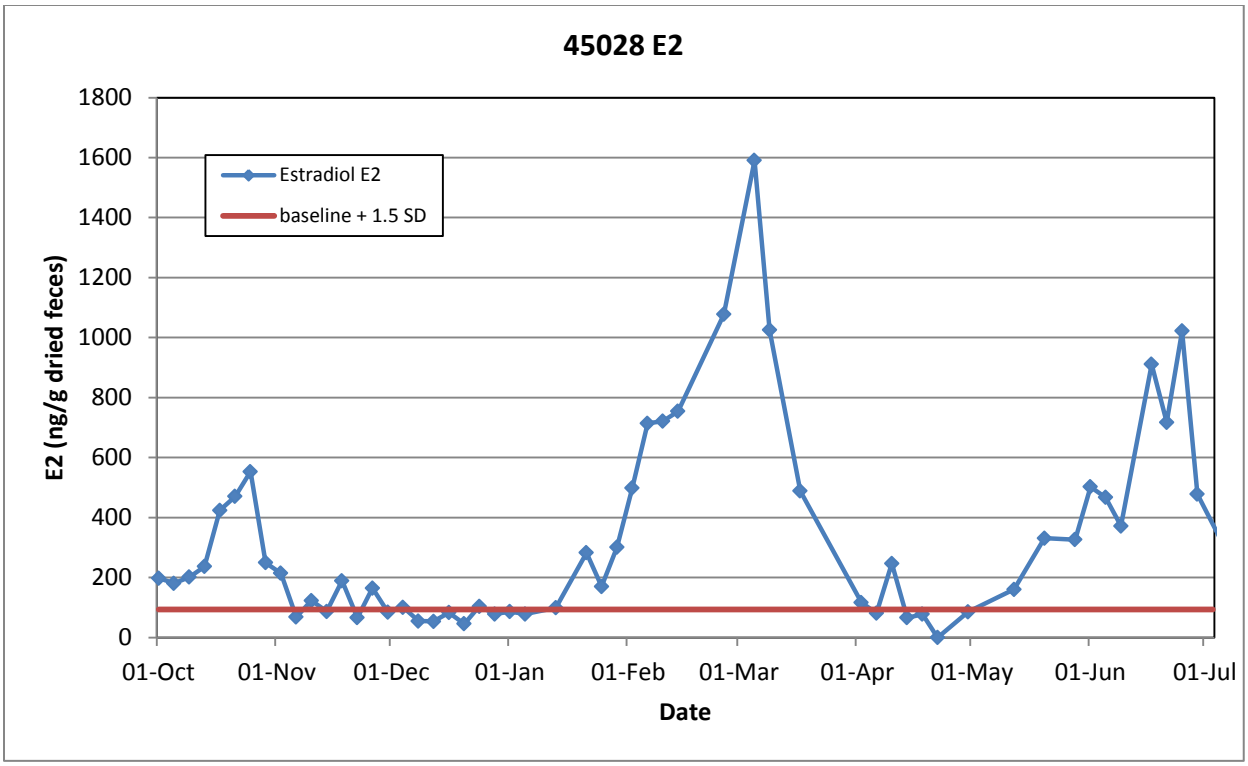

45027 T
Testosterone T
baseline + 1.5 SD
T (ng/g dried feces)
3000
2500
2000
1500
1000
500
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

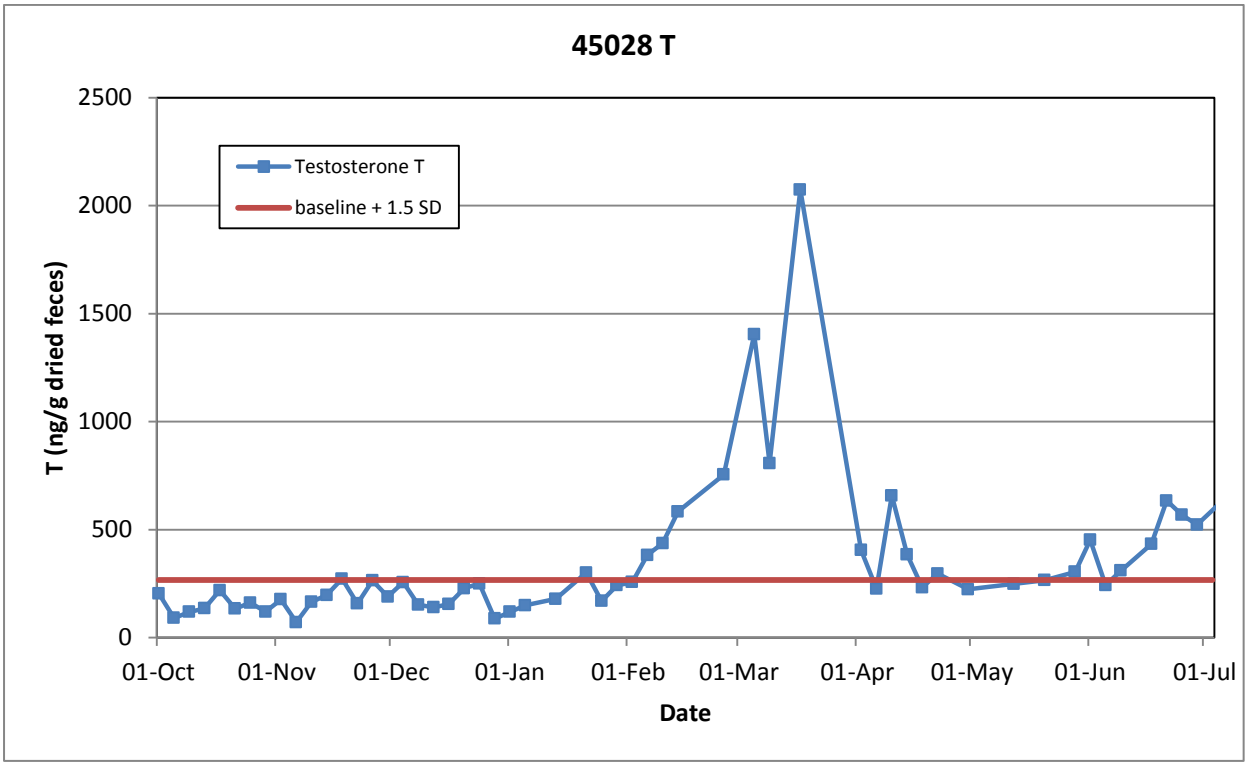

45028
2500
2000
1500
1000
500
0
25000
20000
15000
10000
5000
0
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

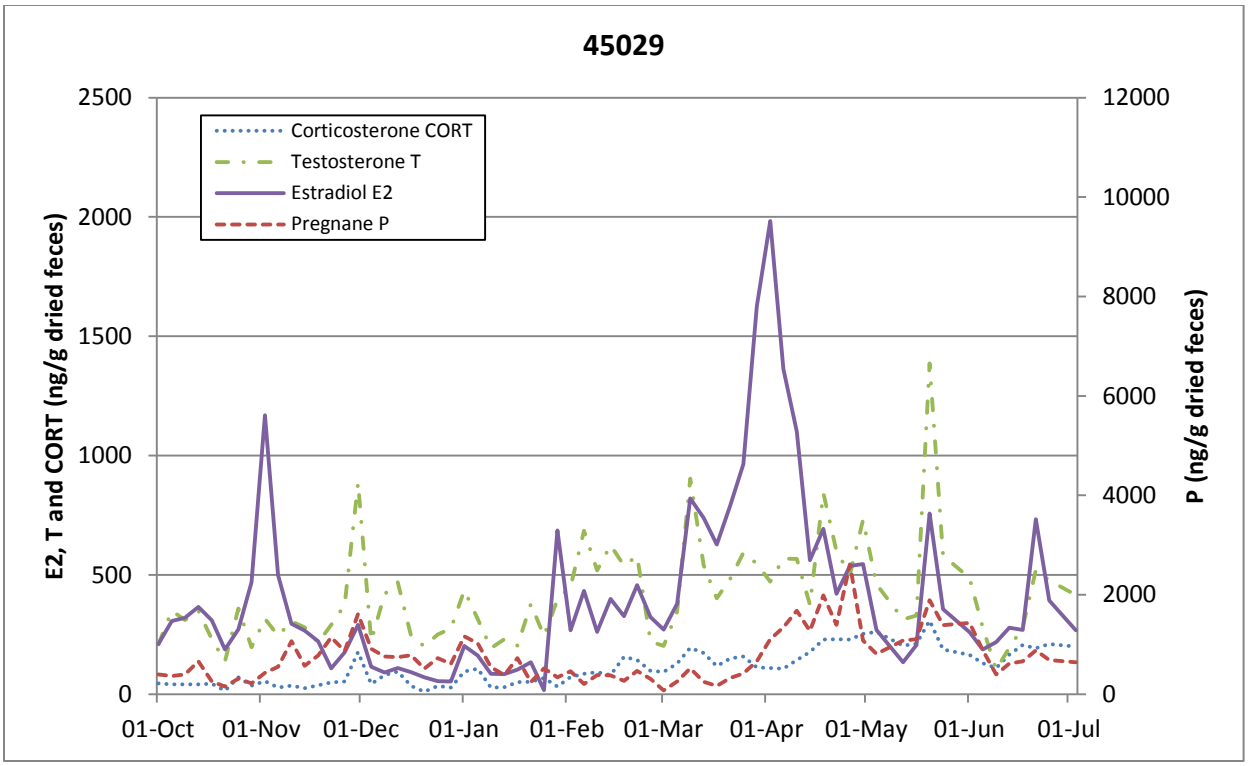

45028 Follicular changes
Length L
Width W
Surface Area SA
1.2
1
0.8
0.6
0.4
0.2
0
L, W (cm) & SA (cm²)
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

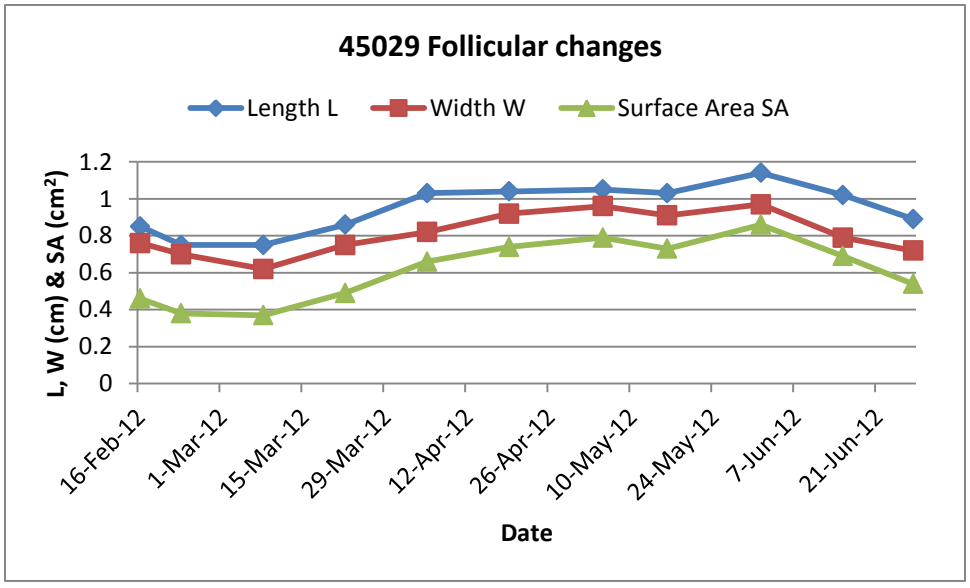

45028 CORT
900
800
700
600
500
400
300
200
100
0
CORT (ng/g dried feces)
Corticosterone CORT
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

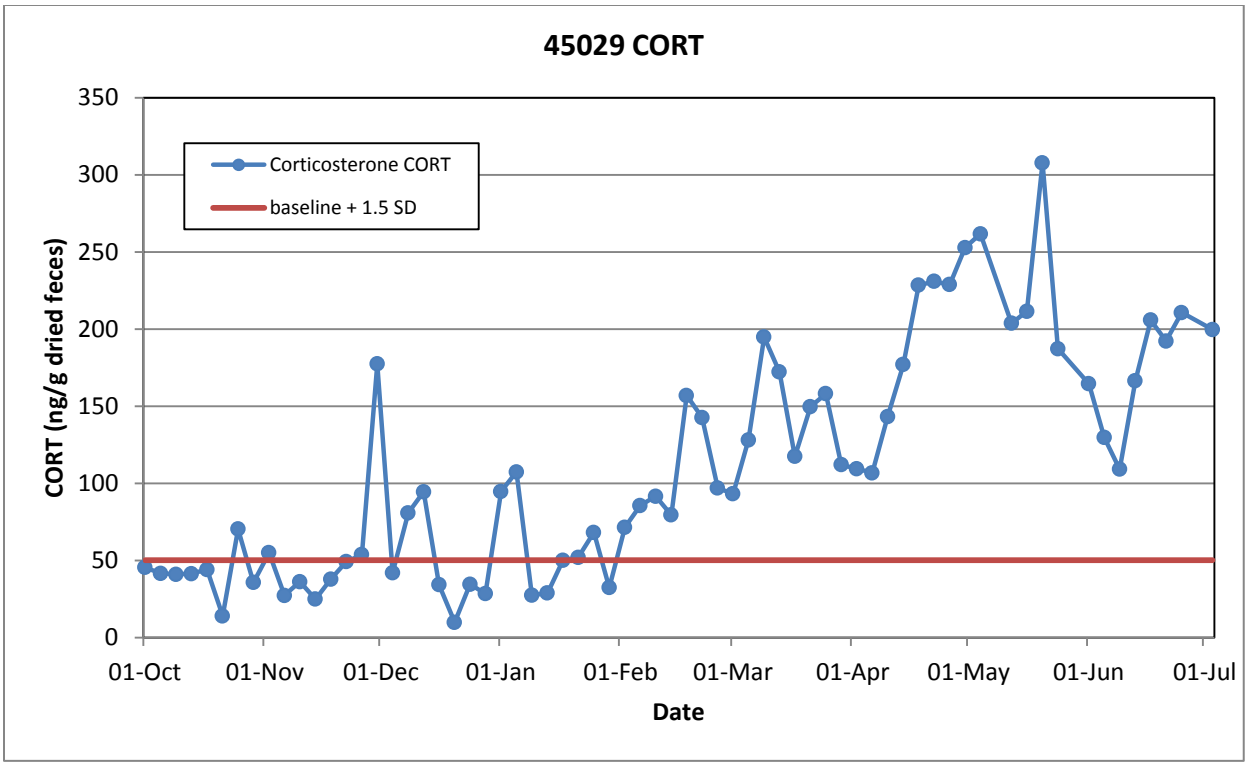

45028 P
2500
2000
1500
1000
500
0
P (ng/g dried feces)
Pregnane P
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

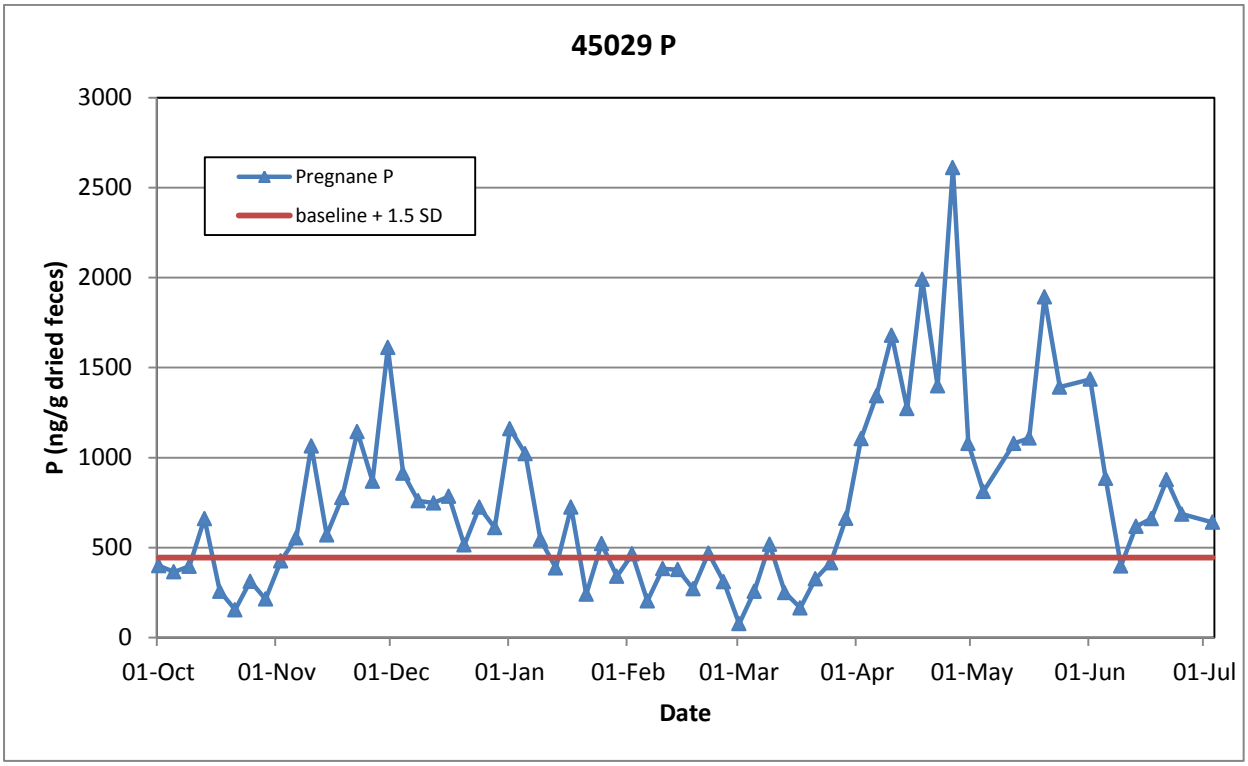

45028 E2
1800
1600
1400
1200
1000
800
600
400
200
0
E2 (ng/g dried feces)
Estradiol E2
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

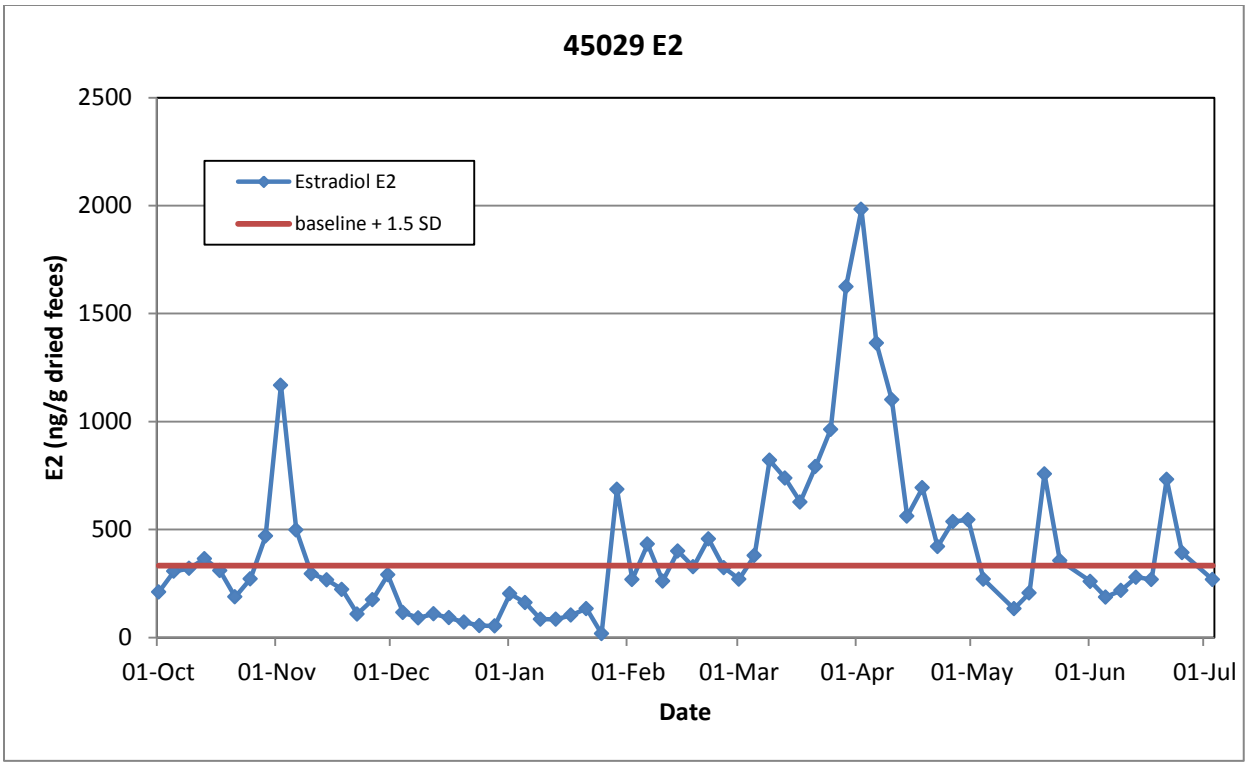

45028 T
2500
2000
1500
1000
500
0
T (ng/g dried feces)
Testosterone T
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

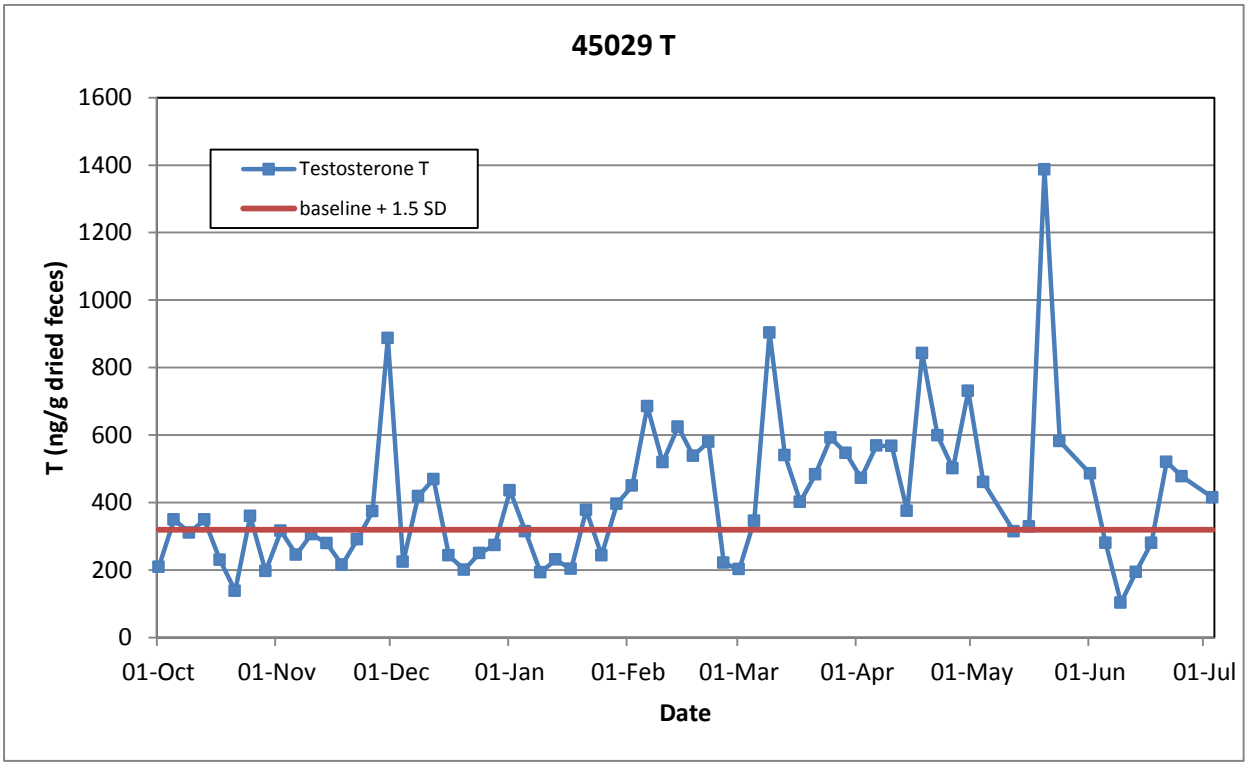

45029
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
2500
2000
1500
1000
500
0
12000
10000
8000
6000
4000
2000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul

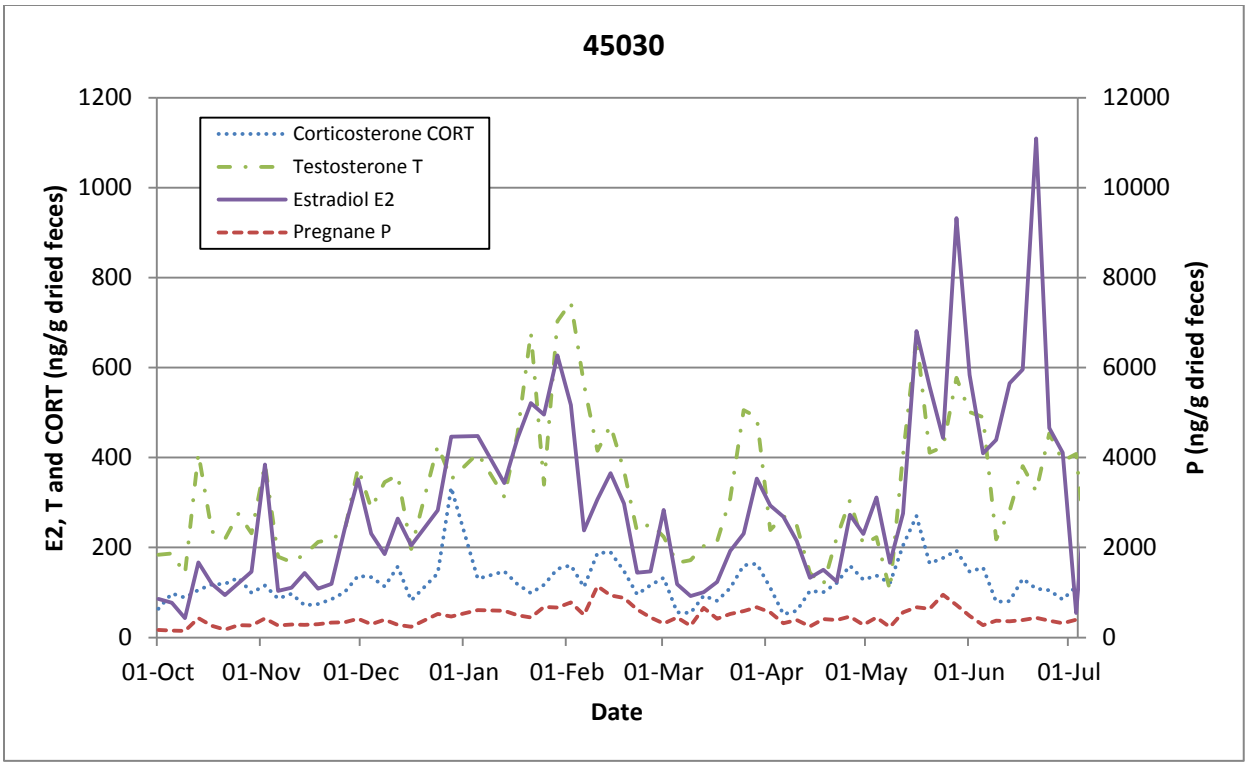

45029 Follicular changes
Length L
Width W
Surface Area SA
L, W (cm) & SA (cm²)
1.2
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

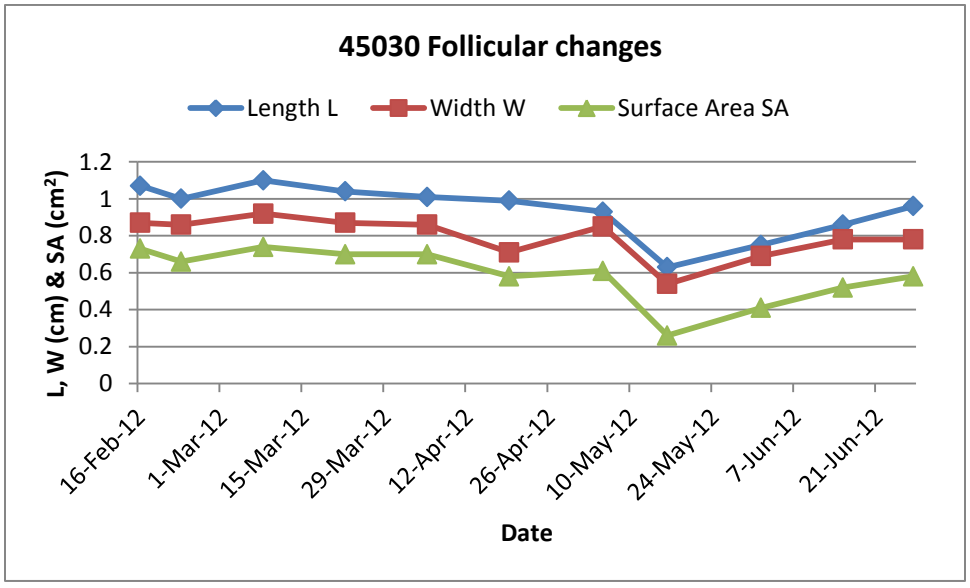

45029 CORT
Corticosterone CORT
baseline + 1.5 SD
CORT (ng/g dried feces)
350
300
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

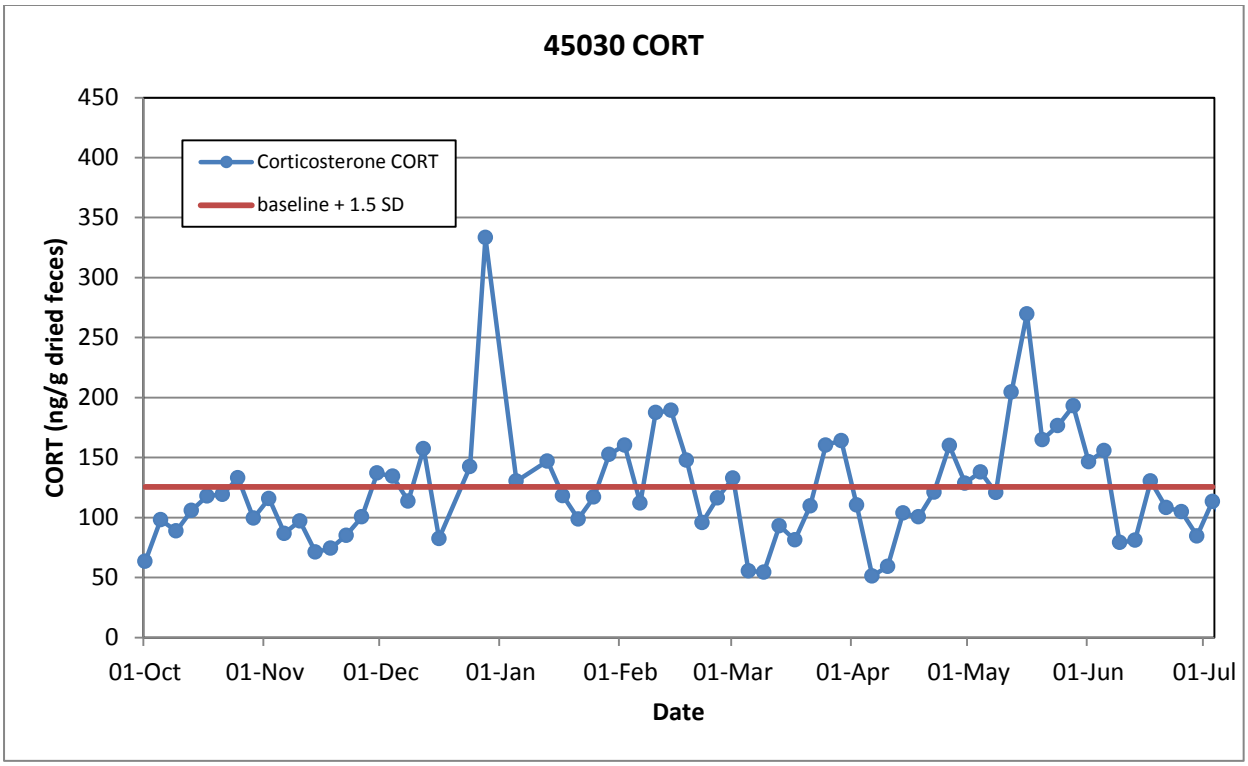

45029 P
Pregnane P
baseline + 1.5 SD
P (ng/g dried feces)
3000
2500
2000
1500
1000
500
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

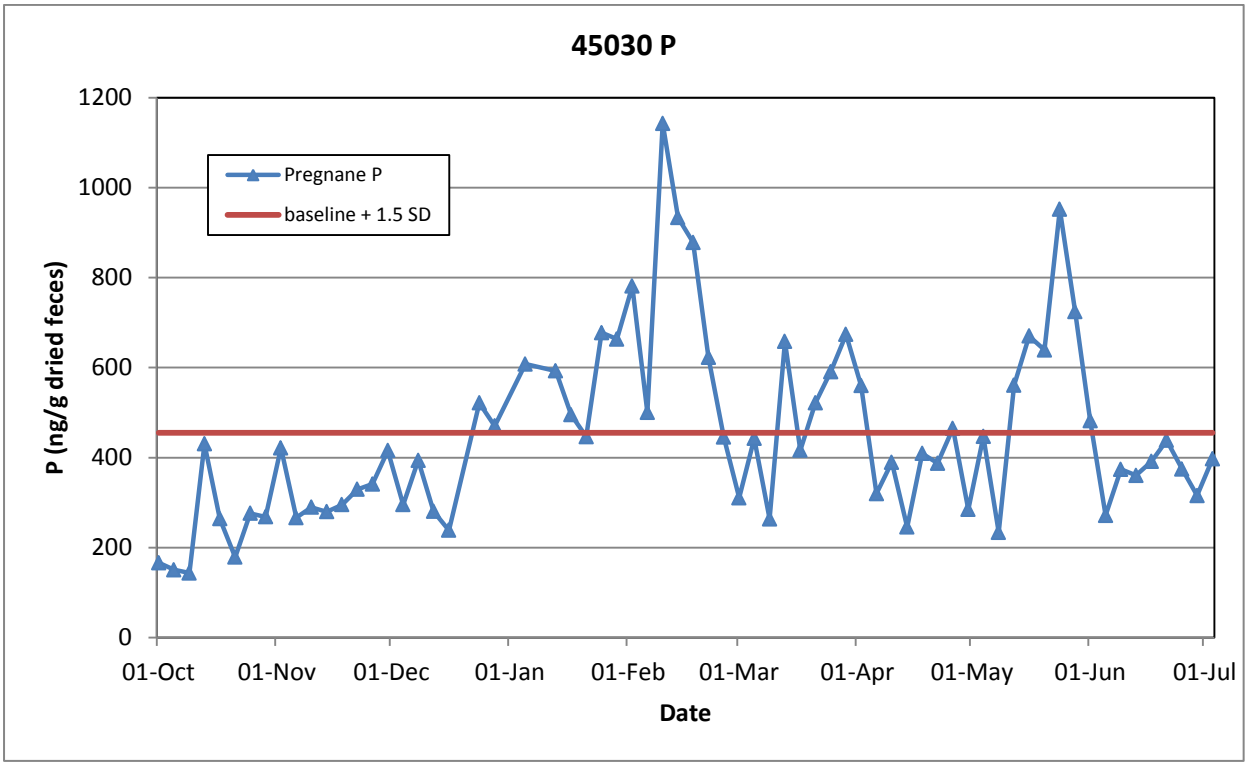

45029 E2
2500
2000
1500
1000
500
0
E2 (ng/g dried feces)
Estradiol E2
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

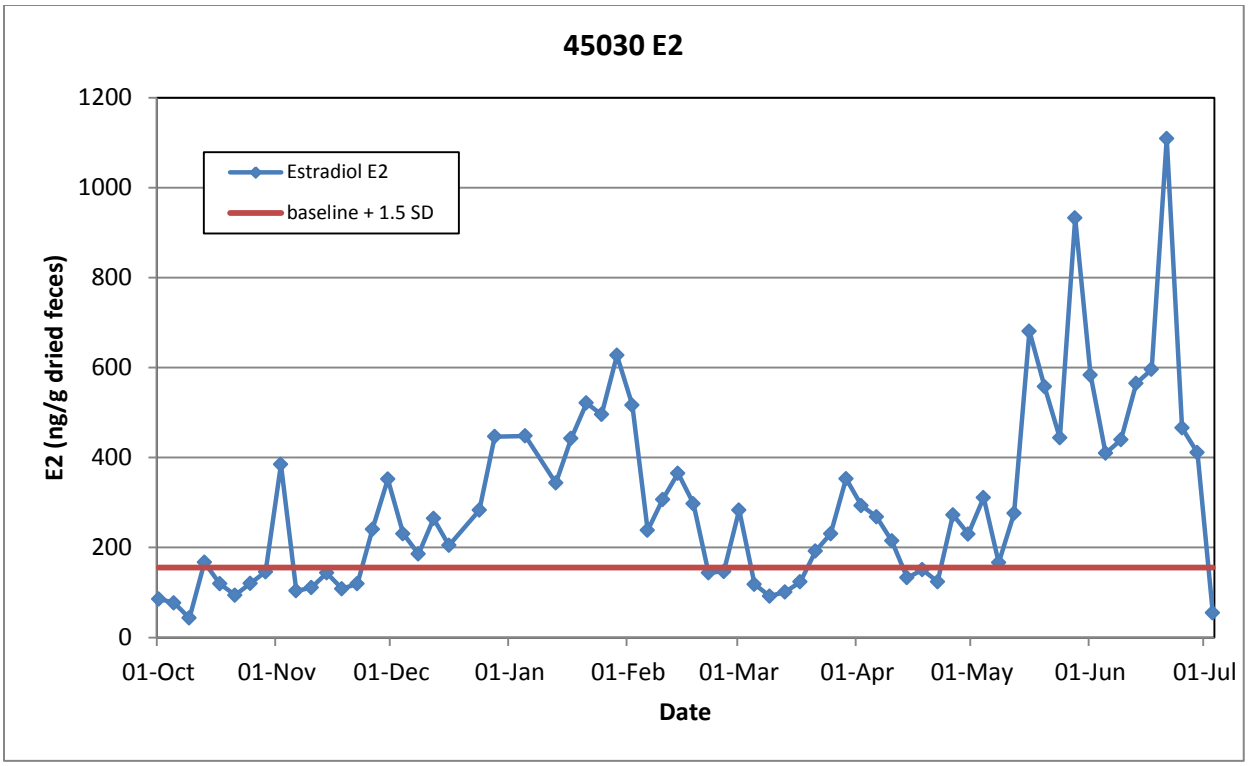

45029 T
1600
1400
1200
1000
800
600
400
200
0
T (ng/g dried feces)
Testosterone T
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

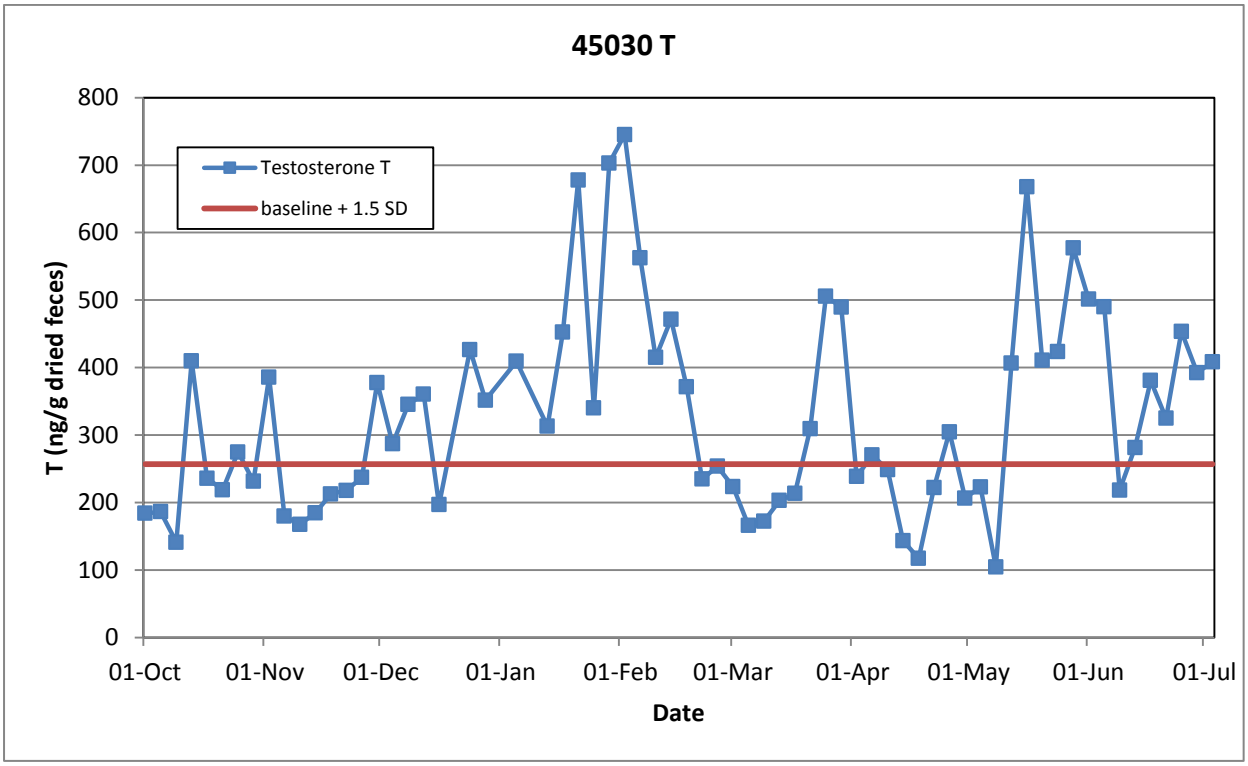

45030
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
1200
1000
800
600
400
200
0
12000
10000
8000
6000
4000
2000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

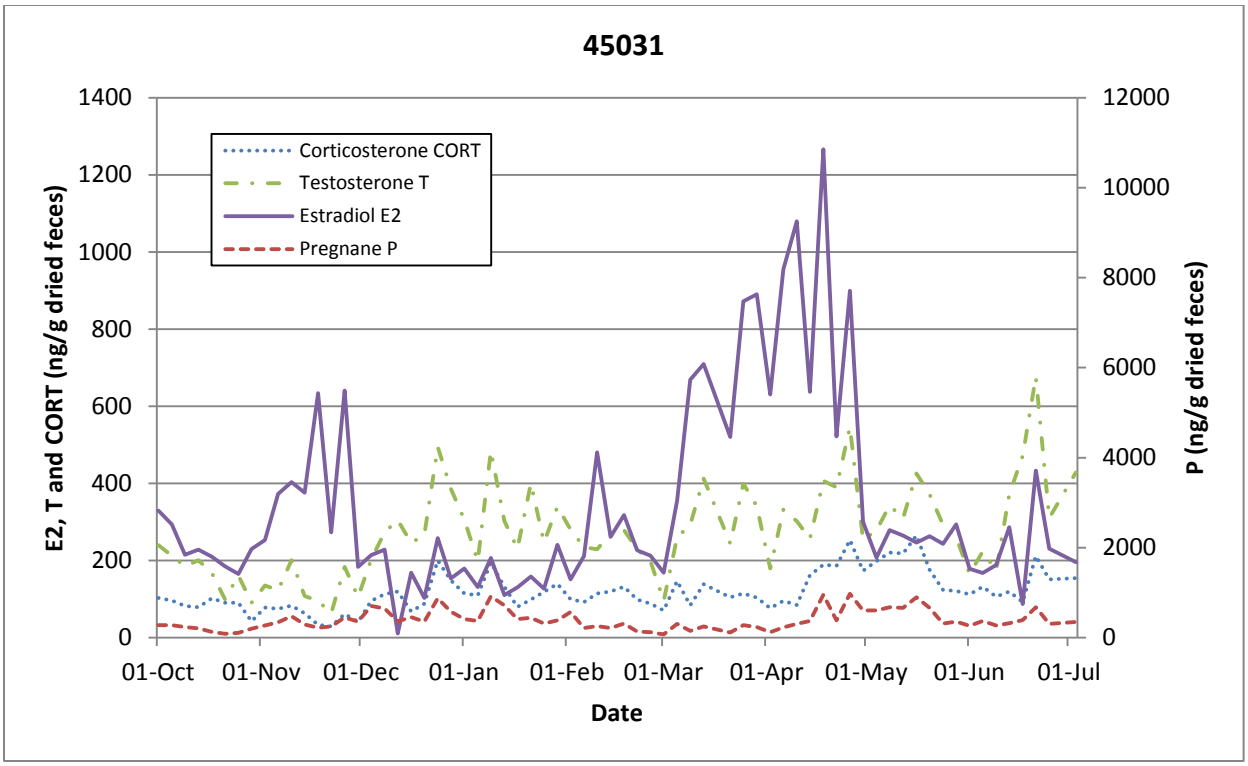

45030 Follicular changes
Length L
Width W
Surface Area SA
L, W (cm) & SA (cm²)
1.2
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

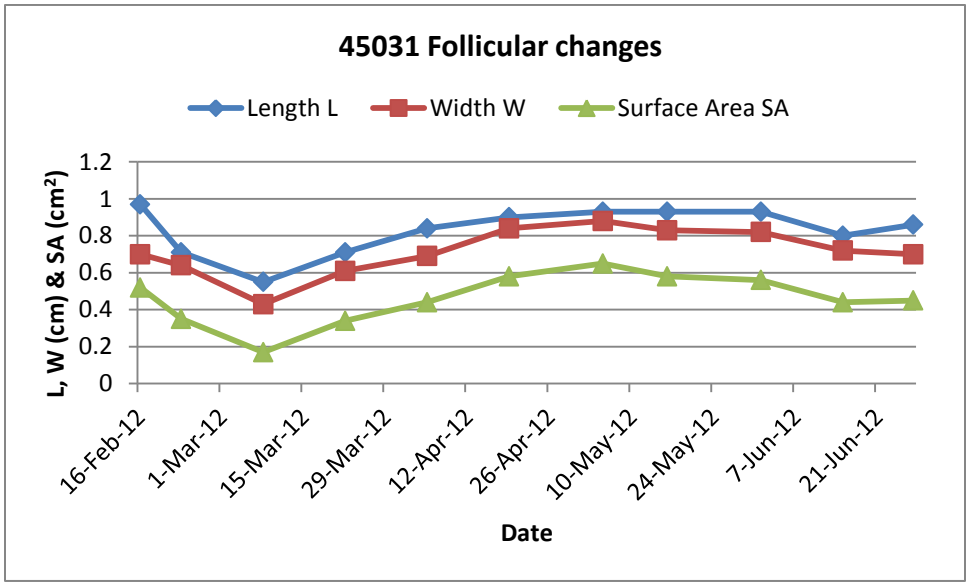

45030 CORT
Corticosterone CORT
baseline + 1.5 SD
CORT (ng/g dried feces)
450
400
350
300
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

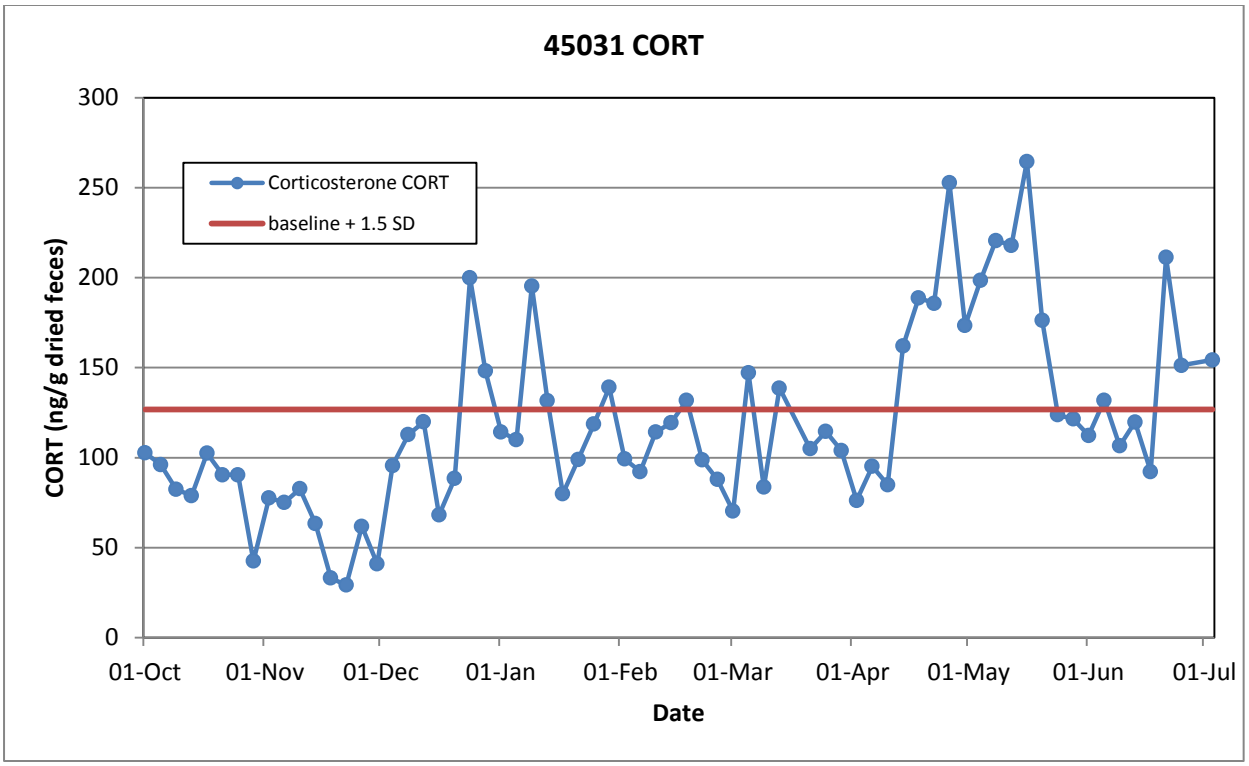

45030 P
Pregnane P
baseline + 1.5 SD
P (ng/g dried feces)
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

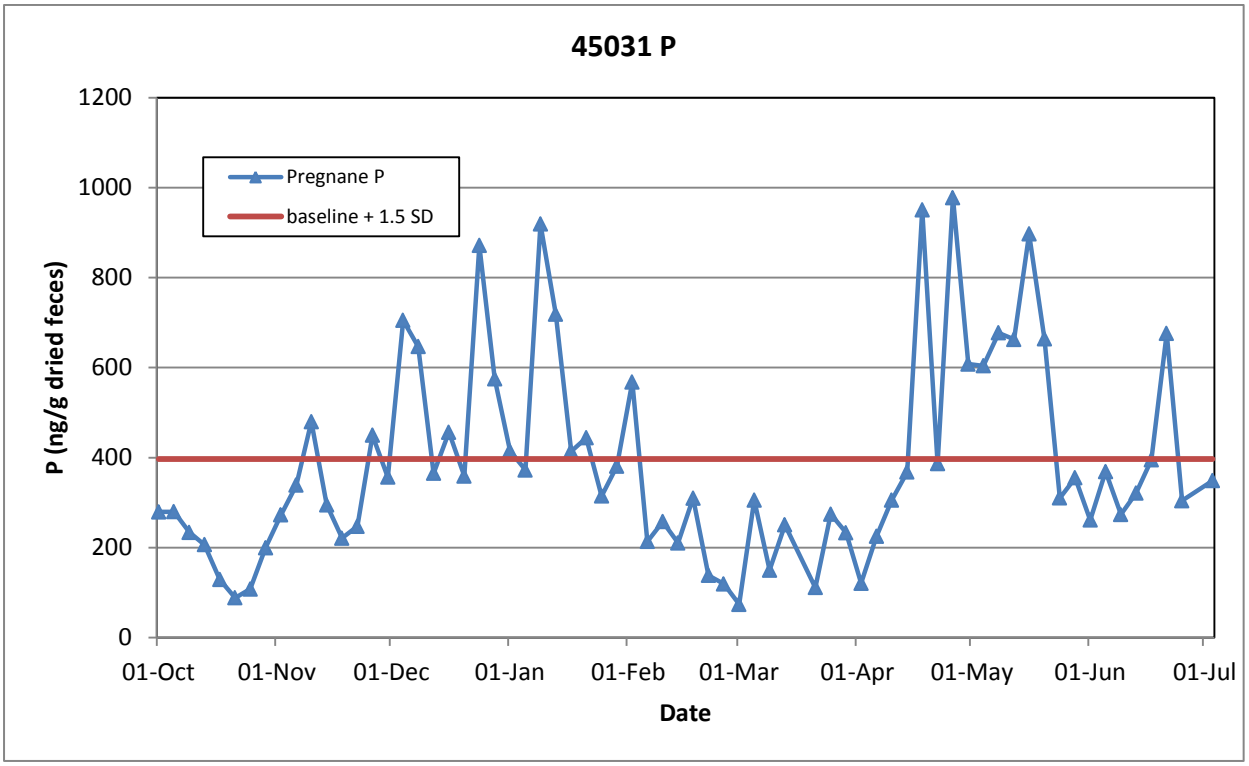

45030 E2
1200
1000
800
600
400
200
0
E2 (ng/g dried feces)
Estradiol E2
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

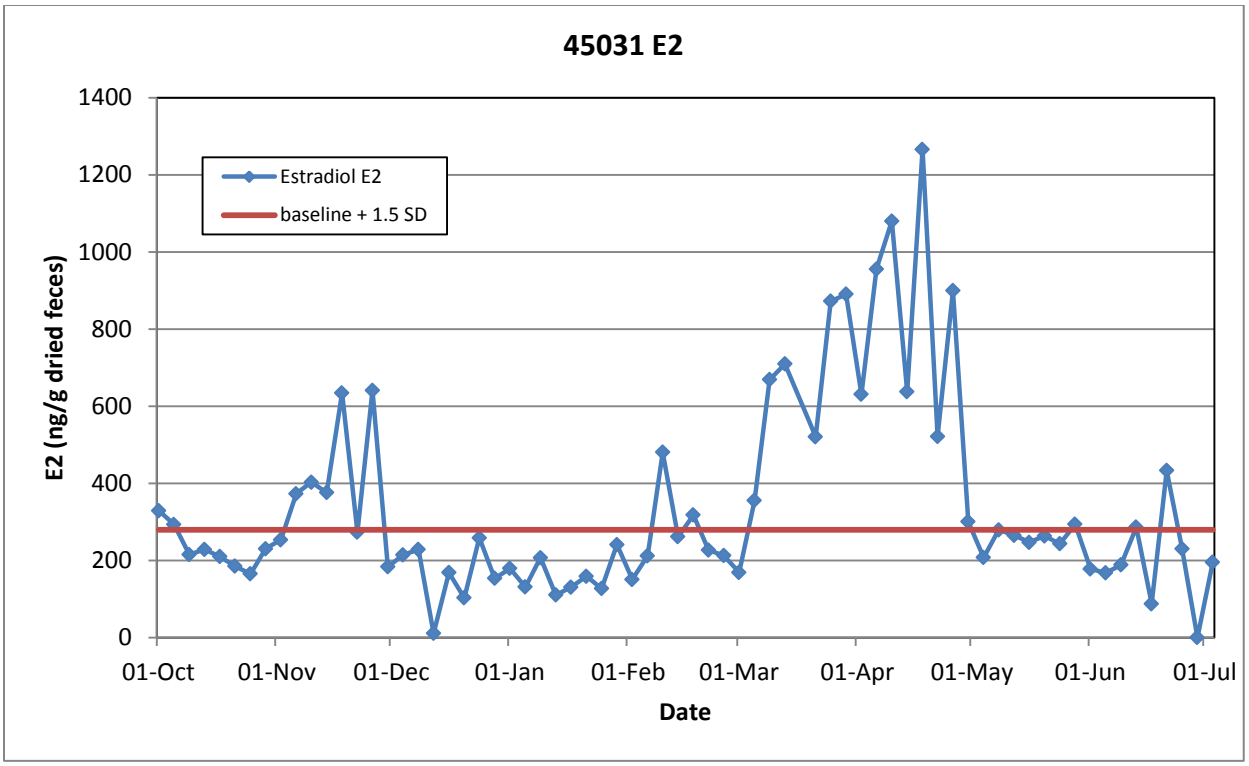

45030 T
800
700
600
500
400
300
200
100
0
T (ng/g dried feces)
Testosterone T
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

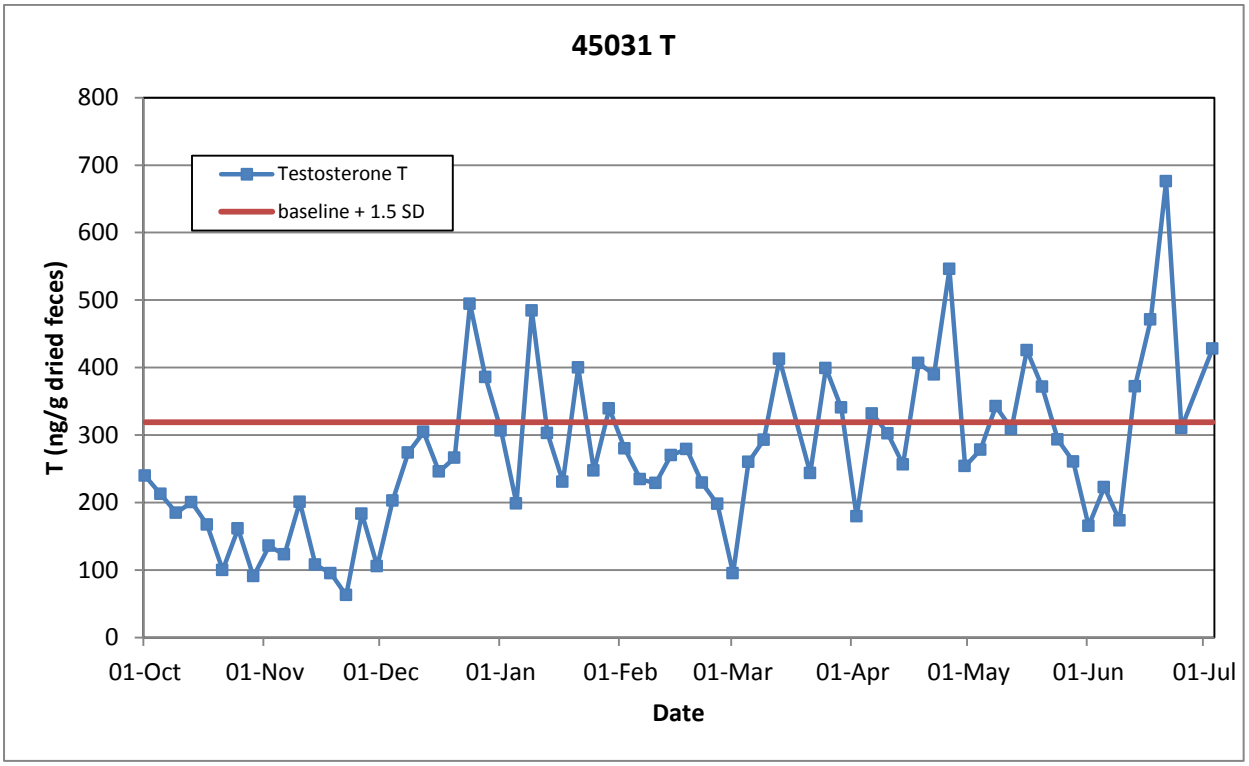

45031
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
1400
1200
1000
800
600
400
200
0
12000
10000
8000
6000
4000
2000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

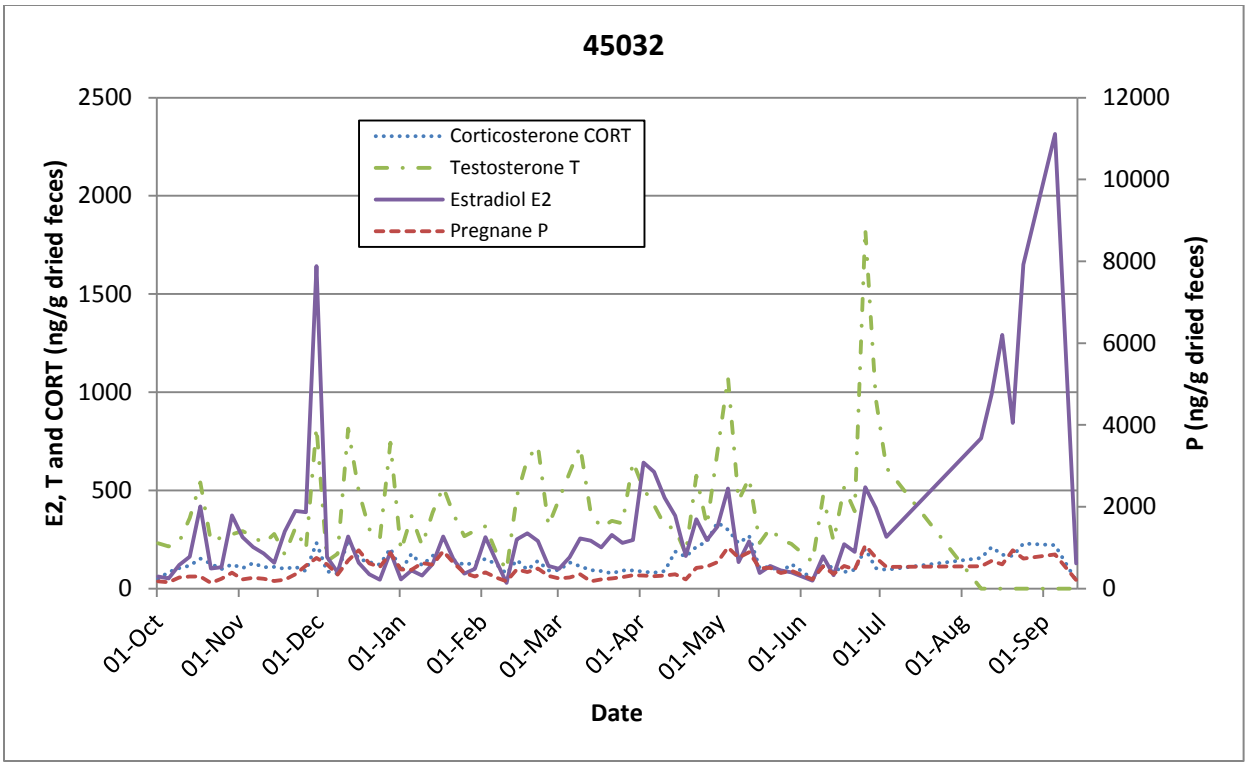

45031 Follicular changes
Length L
Width W
Surface Area SA
L, W (cm) & SA (cm²)
1.2
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

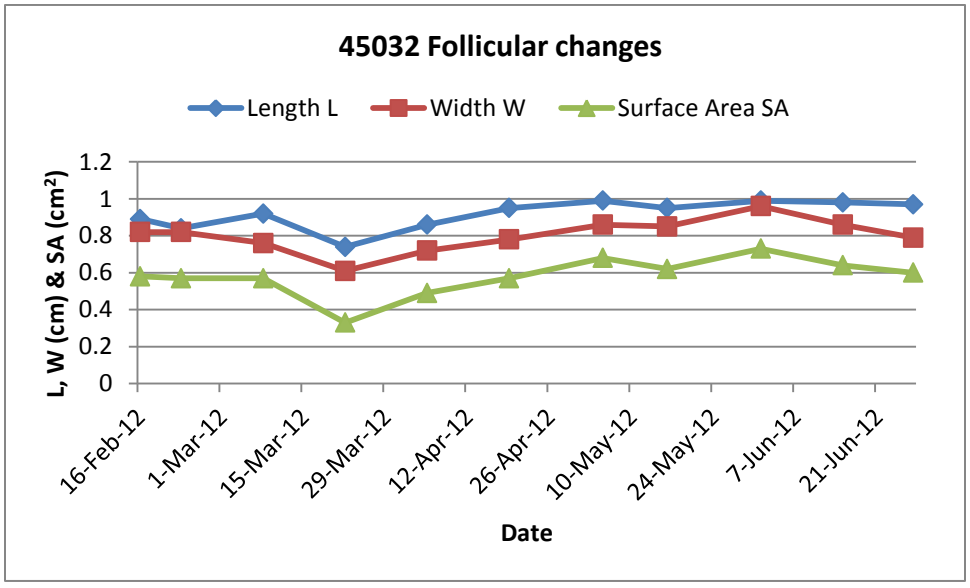

45031 CORT
Corticosterone CORT
baseline + 1.5 SD
CORT (ng/g dried feces)
300
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

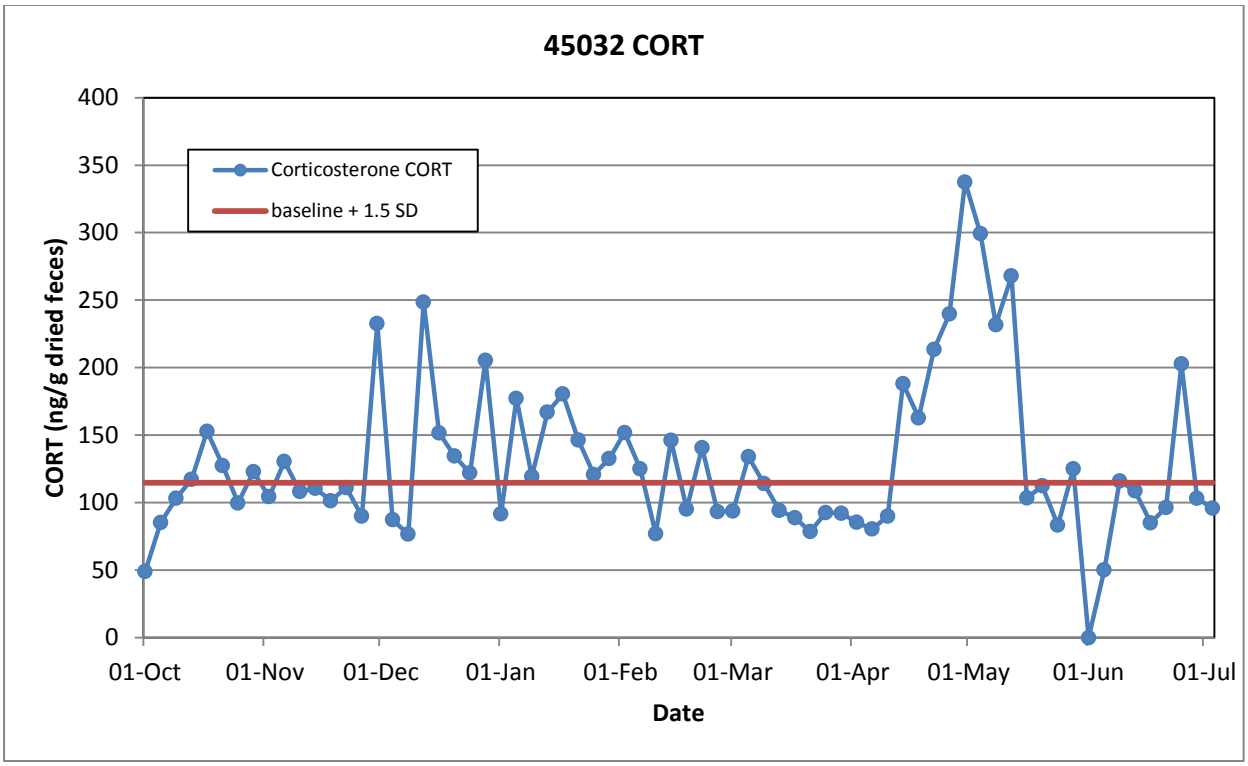

45031 P
Pregnane P
baseline + 1.5 SD
P (ng/g dried feces)
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

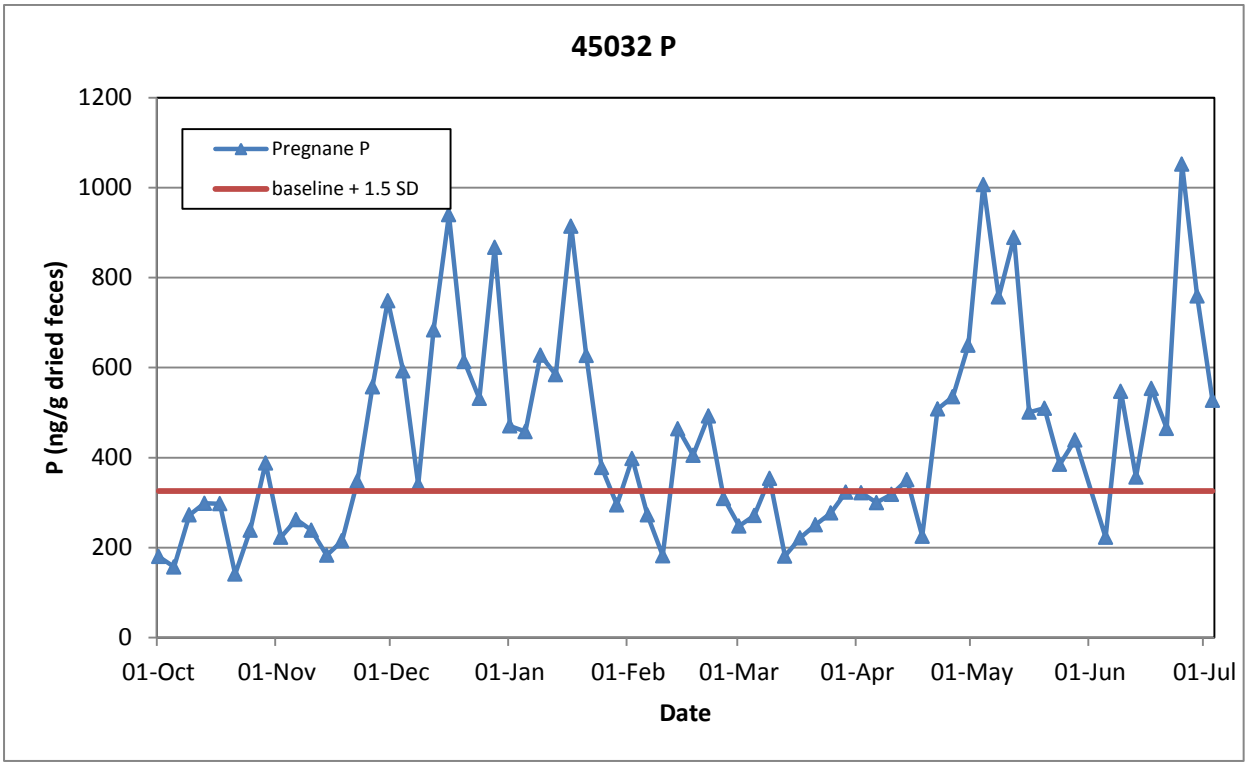

45031 E2
1400
1200
1000
800
600
400
200
0
E2 (ng/g dried feces)
Estradiol E2
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

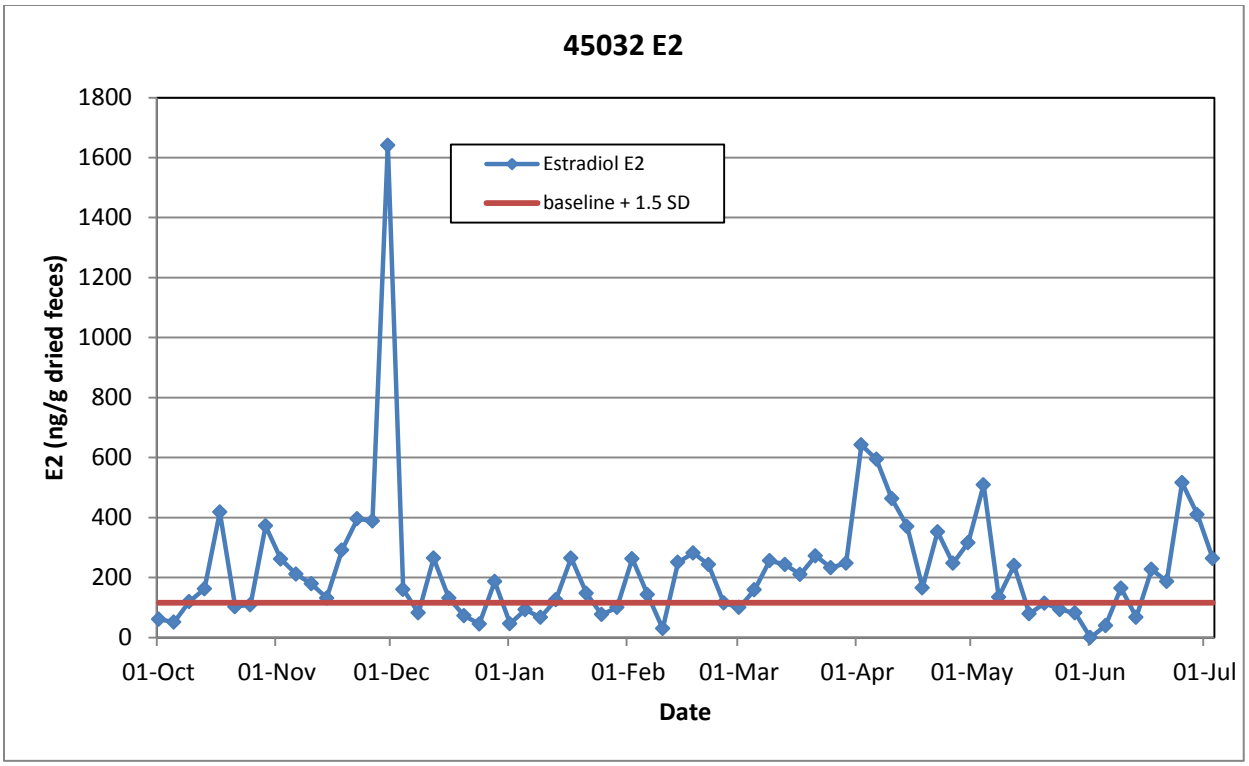

45031 T
800
700
600
500
400
300
200
100
0
T (ng/g dried feces)
Testosterone T
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

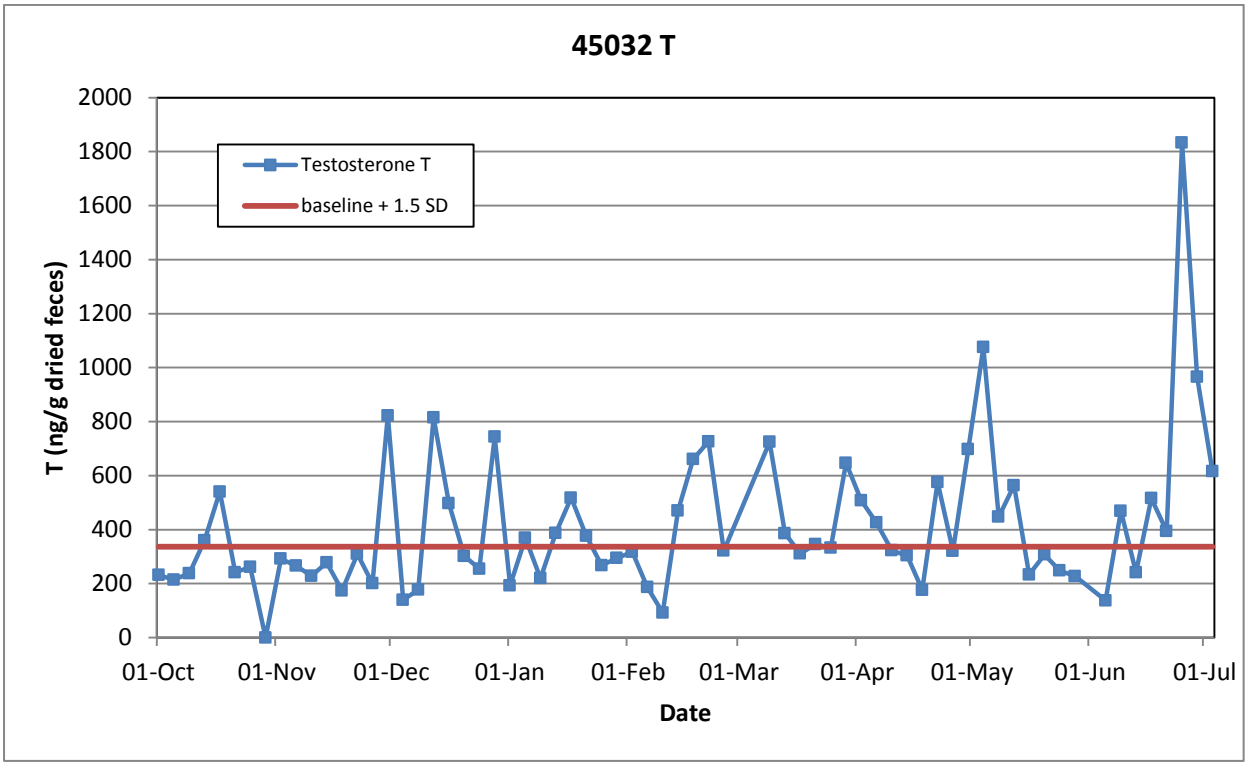

45032
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
2500
2000
1500
1000
500
0
12000
10000
8000
6000
4000
2000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
01-Aug
01-Sep
Date

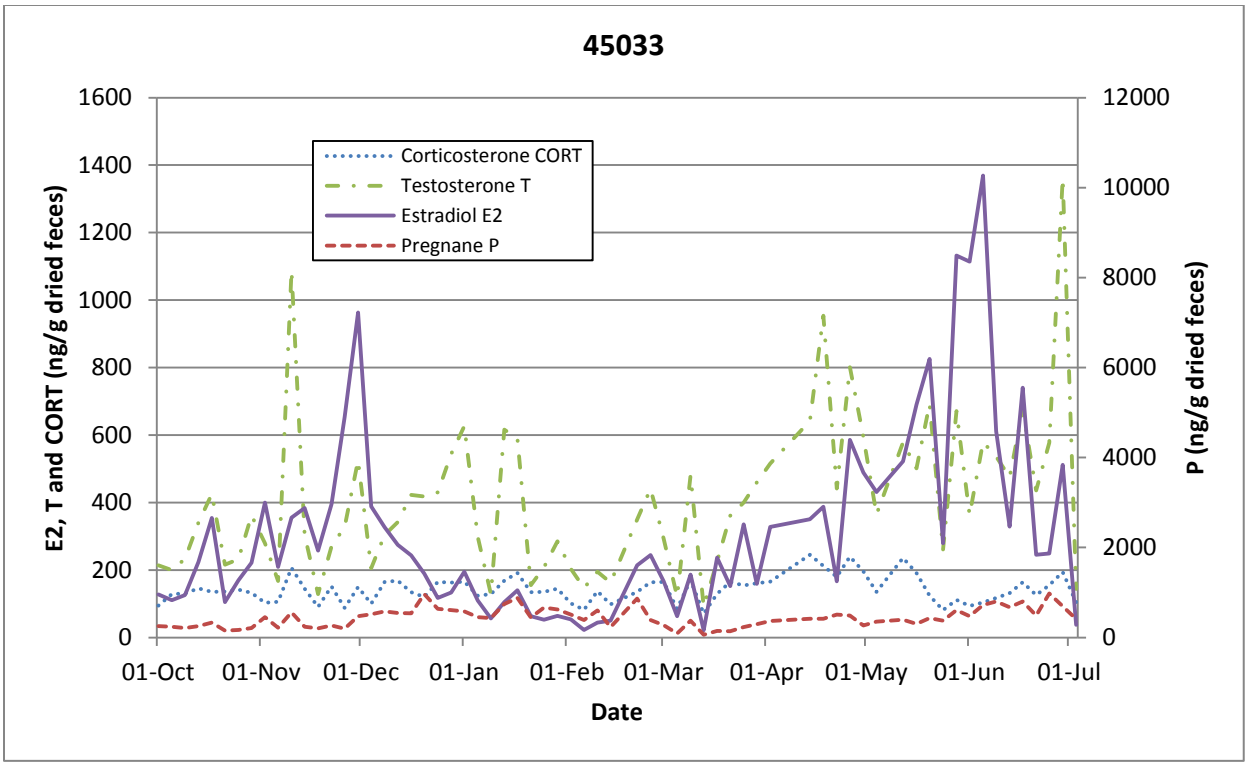

45032 Follicular changes
Length L
Width W
Surface Area SA
L, W (cm) & SA (cm²)
1.2
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

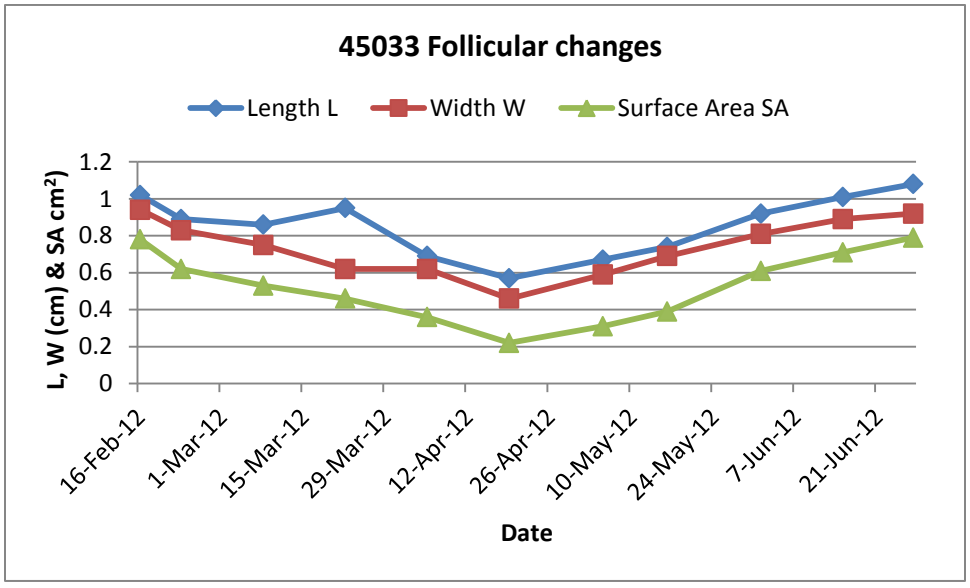

45032 CORT
Corticosterone CORT
baseline + 1.5 SD
CORT (ng/g dried feces)
400
350
300
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

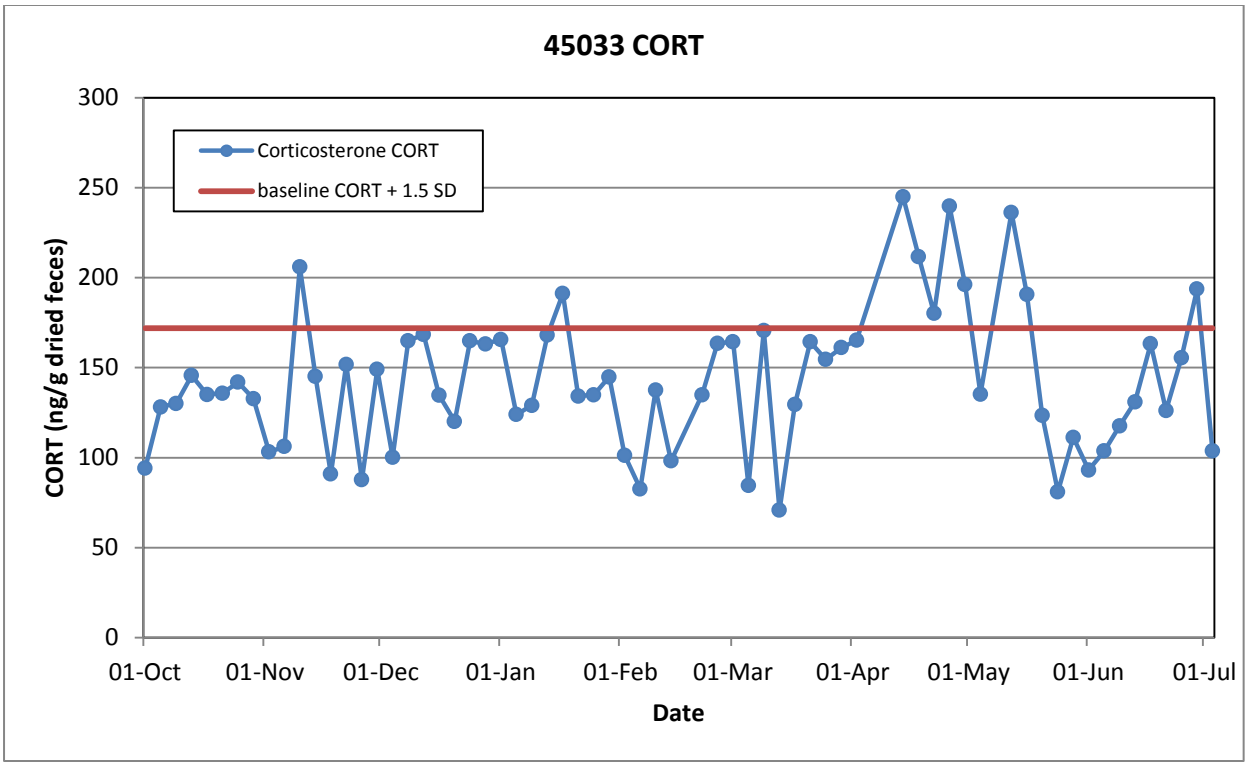

45032 P
Pregnane P
baseline + 1.5 SD
P (ng/g dried feces)
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

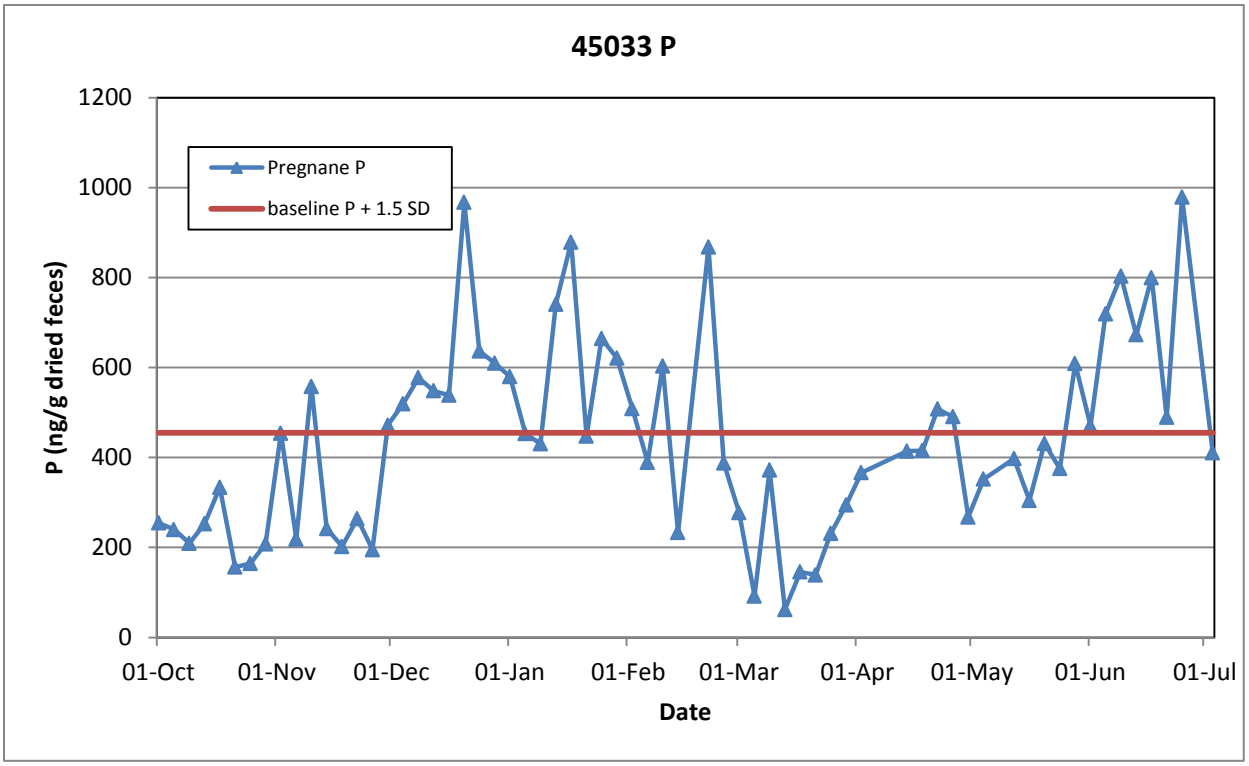

45032 E2
1800
1600
1400
1200
1000
800
600
400
200
0
E2 (ng/g dried feces)
Estradiol E2
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

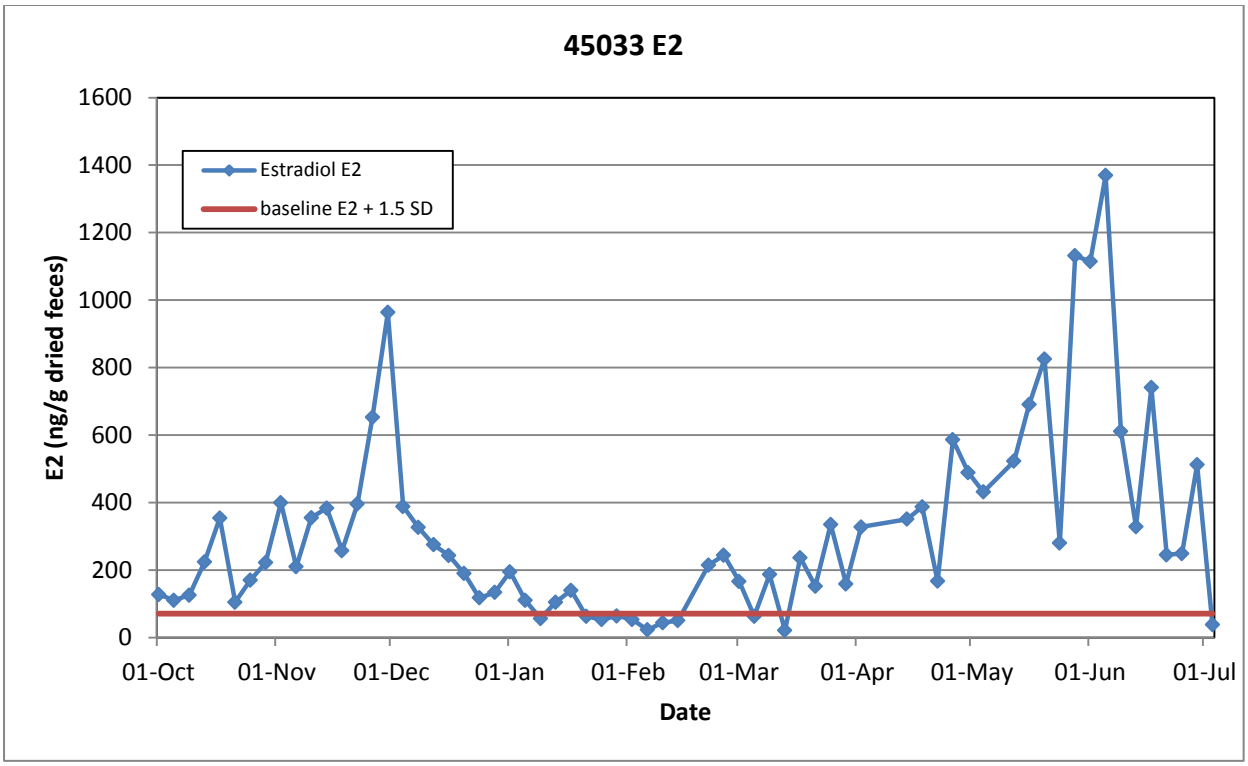

45032 T
2000
1800
1600
1400
1200
1000
800
600
400
200
0
T (ng/g dried feces)
Testosterone T
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

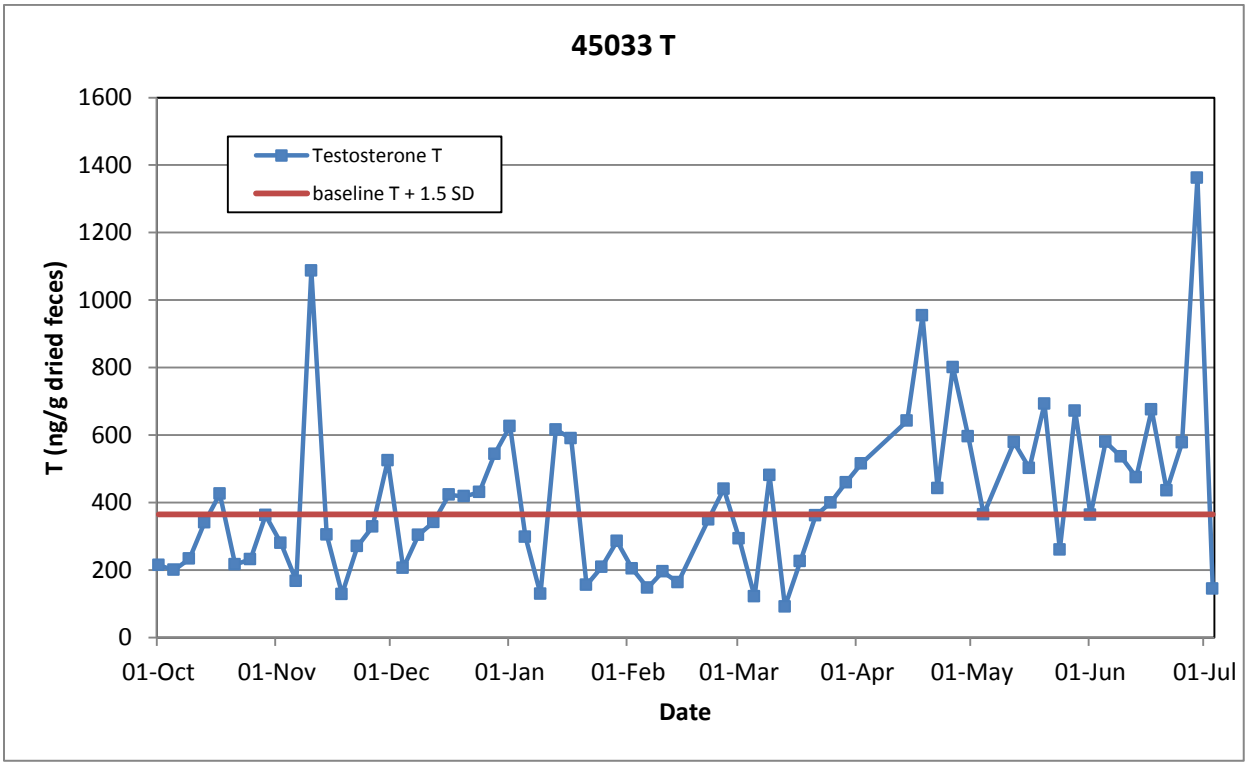

45033
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
1600
1400
1200
1000
800
600
400
200
0
12000
10000
8000
6000
4000
2000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

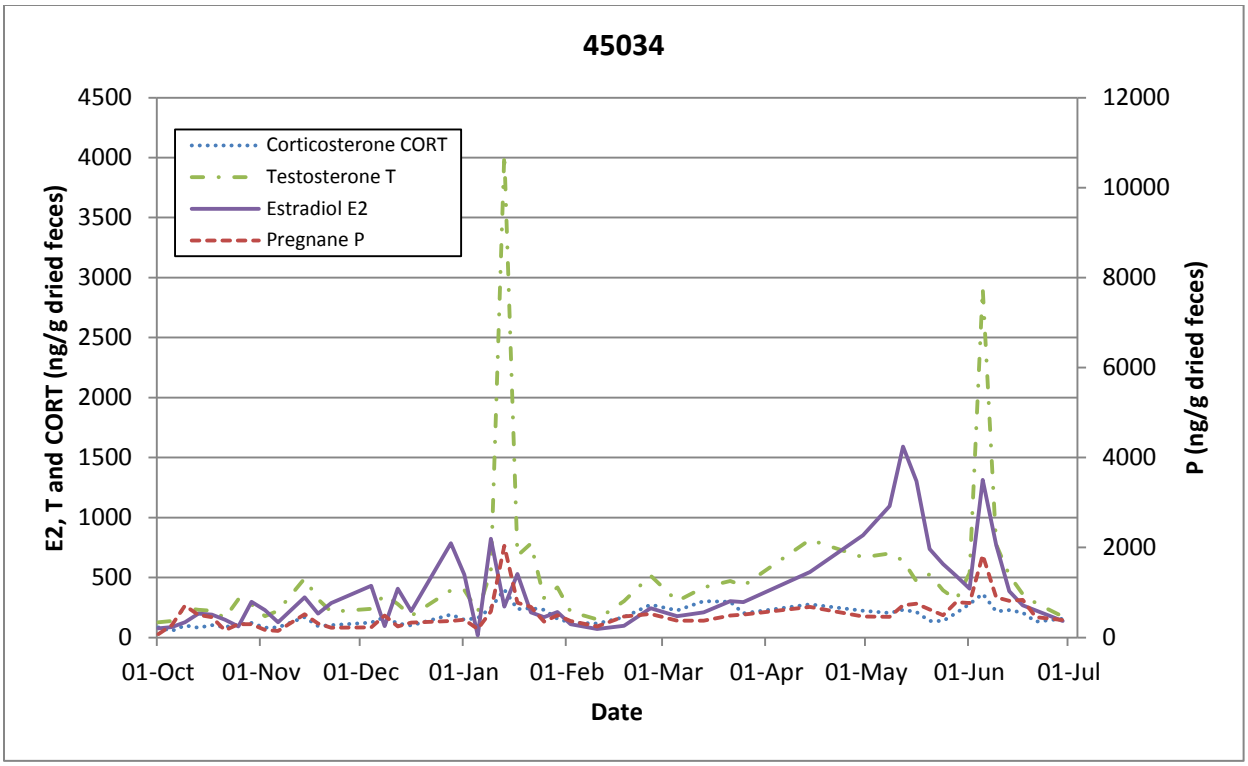

45033 Follicular changes
Length L
Width W
Surface Area SA
L, W (cm) & SA cm²
1.2
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

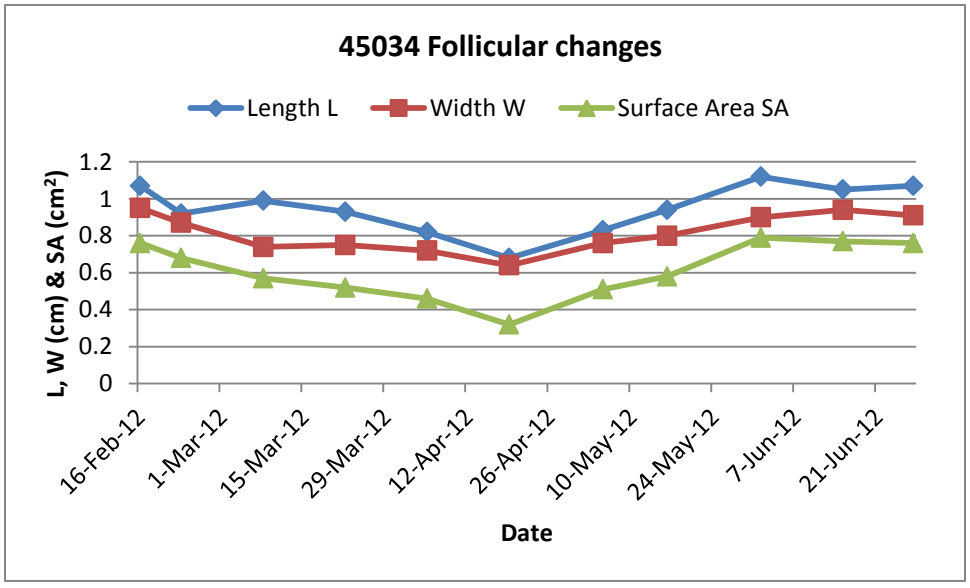

45033 CORT
Corticosterone CORT
baseline CORT + 1.5 SD
CORT (ng/g dried feces)
300
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

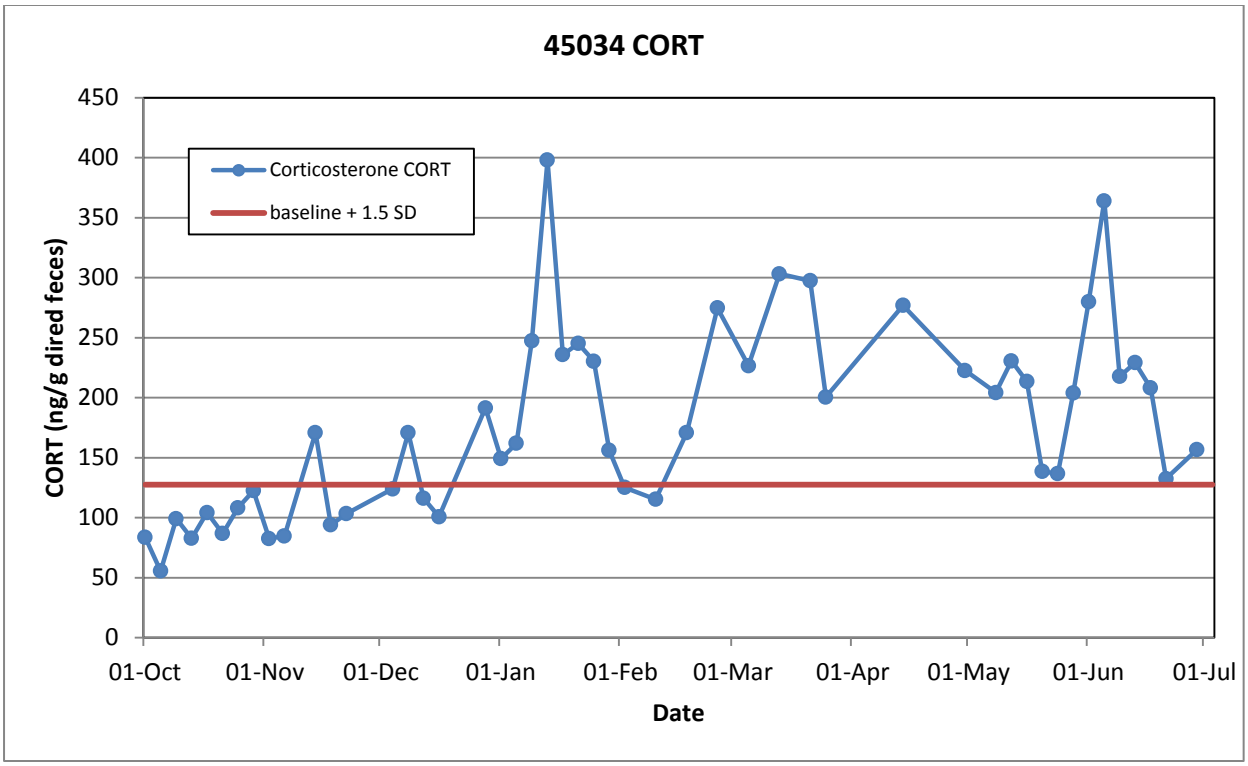

45033 P
Pregnane P
baseline P + 1.5 SD
P (ng/g dried feces)
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

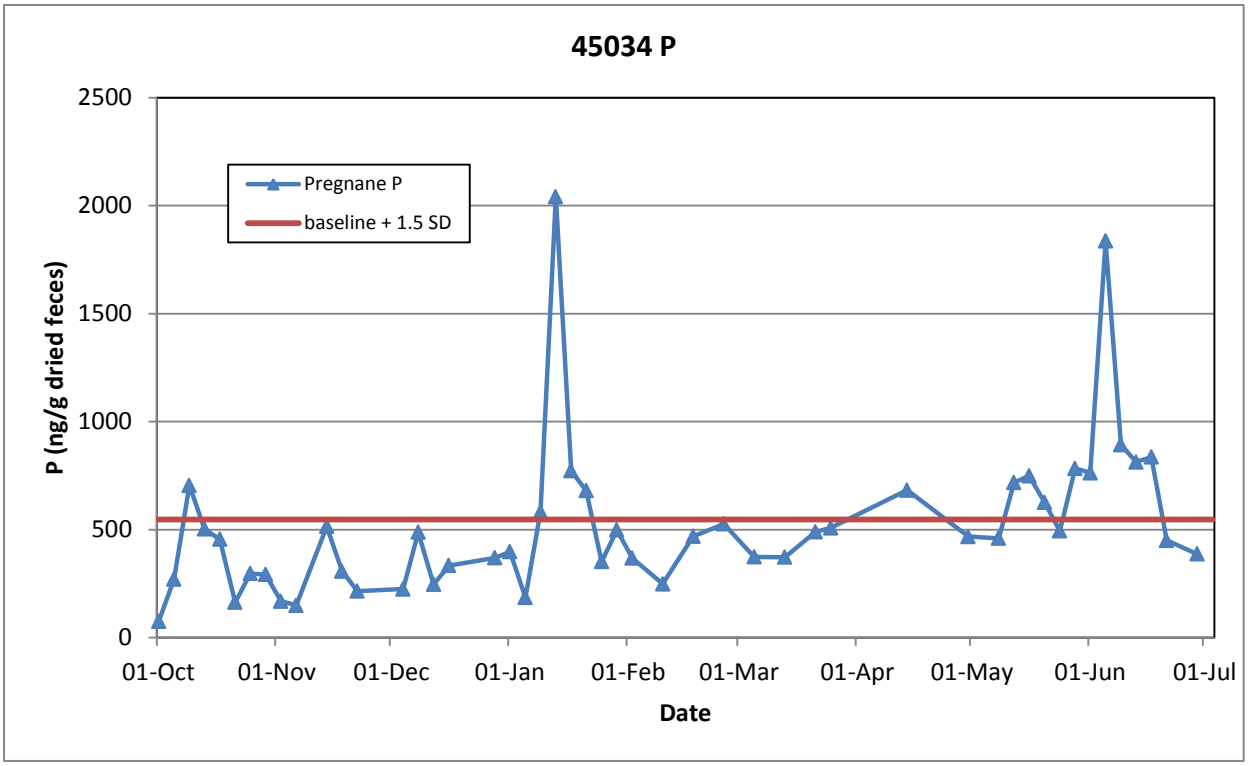

45033 E2
1600
1400
1200
1000
800
600
400
200
0
E2 (ng/g dried feces)
Estradiol E2
baseline E2 + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

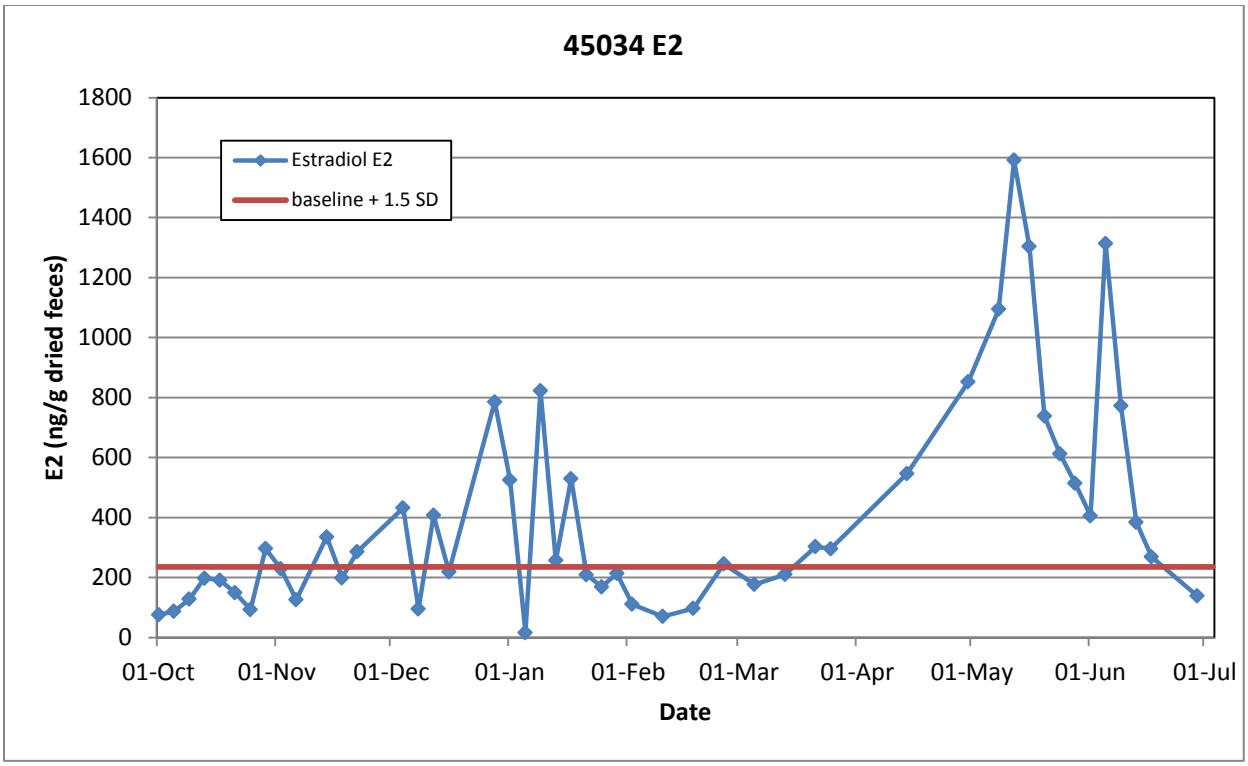

45033 T
1600
1400
1200
1000
800
600
400
200
0
T (ng/g dried feces)
Testosterone T
baseline T + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

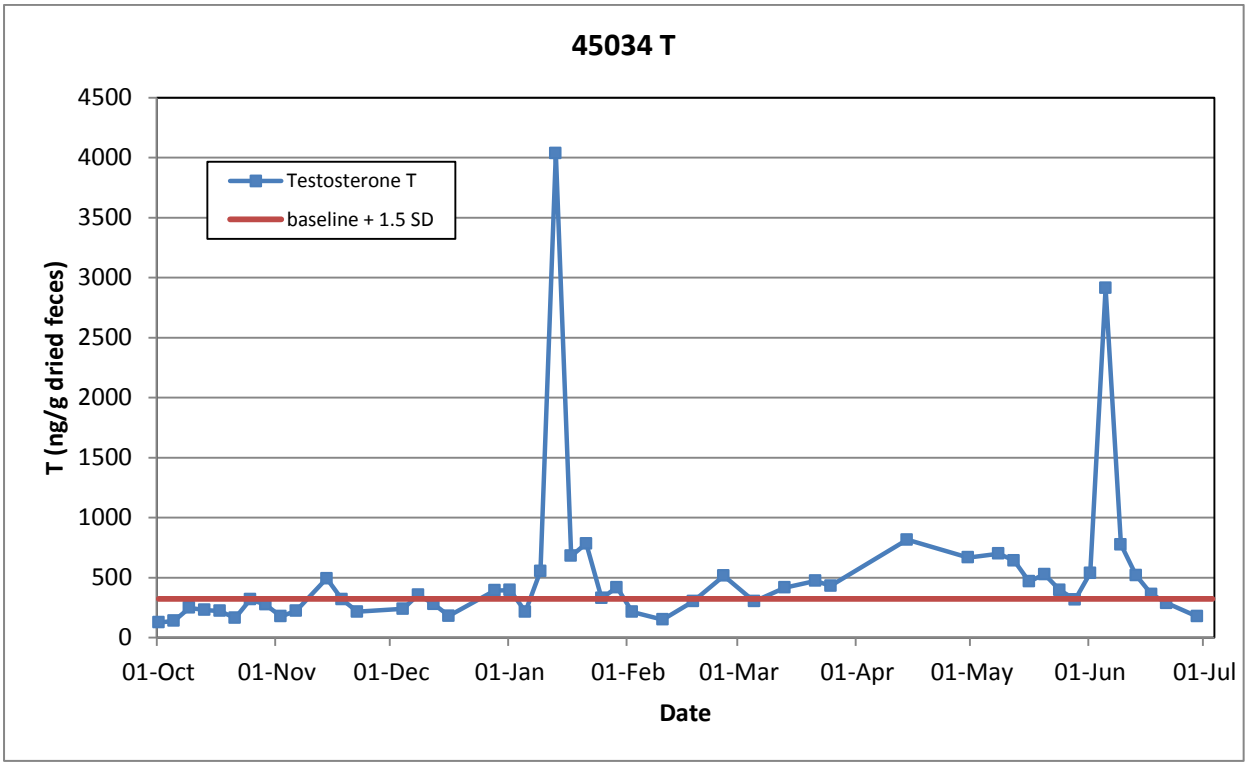

45034
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
12000
10000
8000
6000
4000
2000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

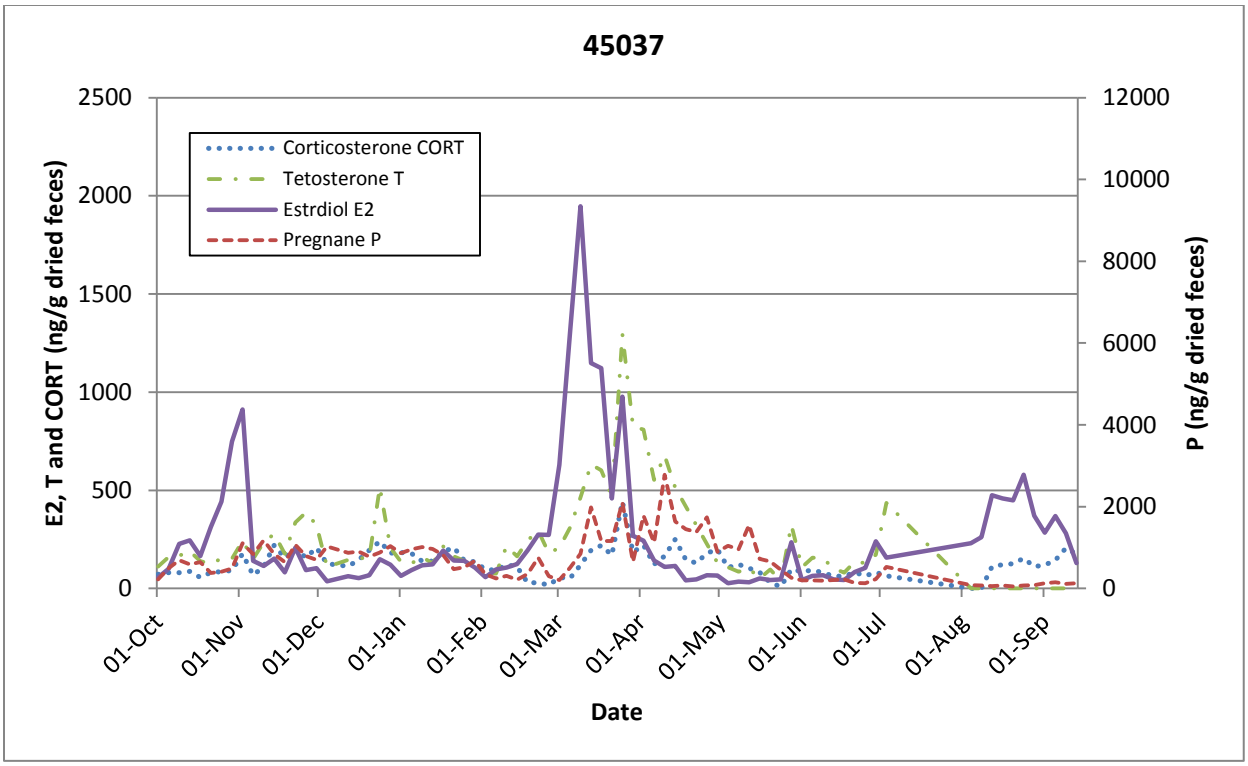

45034 Follicular changes
Length L
Width W
Surface Area SA
L, W (cm) & SA (cm²)
1.2
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

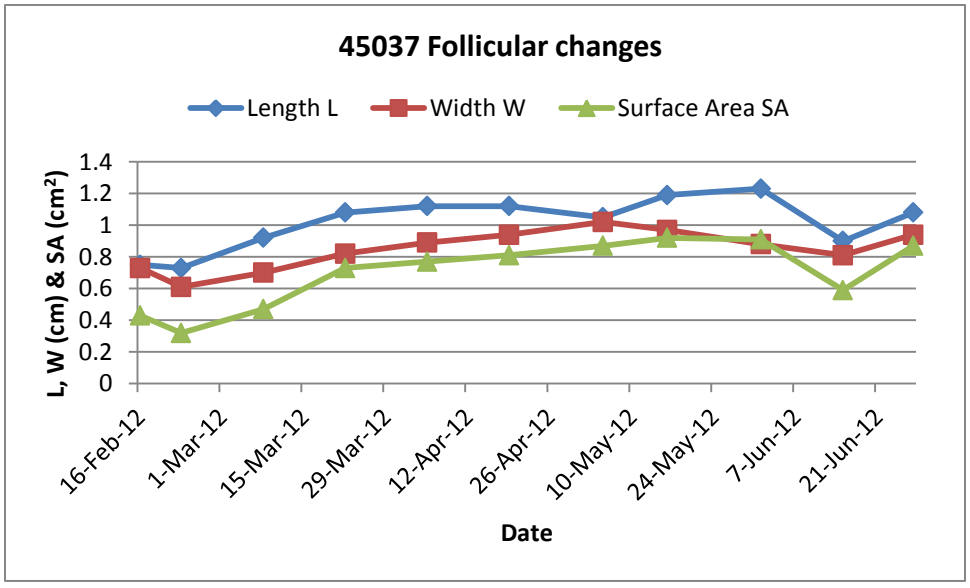

45034 CORT
Corticosterone CORT
baseline + 1.5 SD
CORT (ng/g dired feces)
450
400
350
300
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

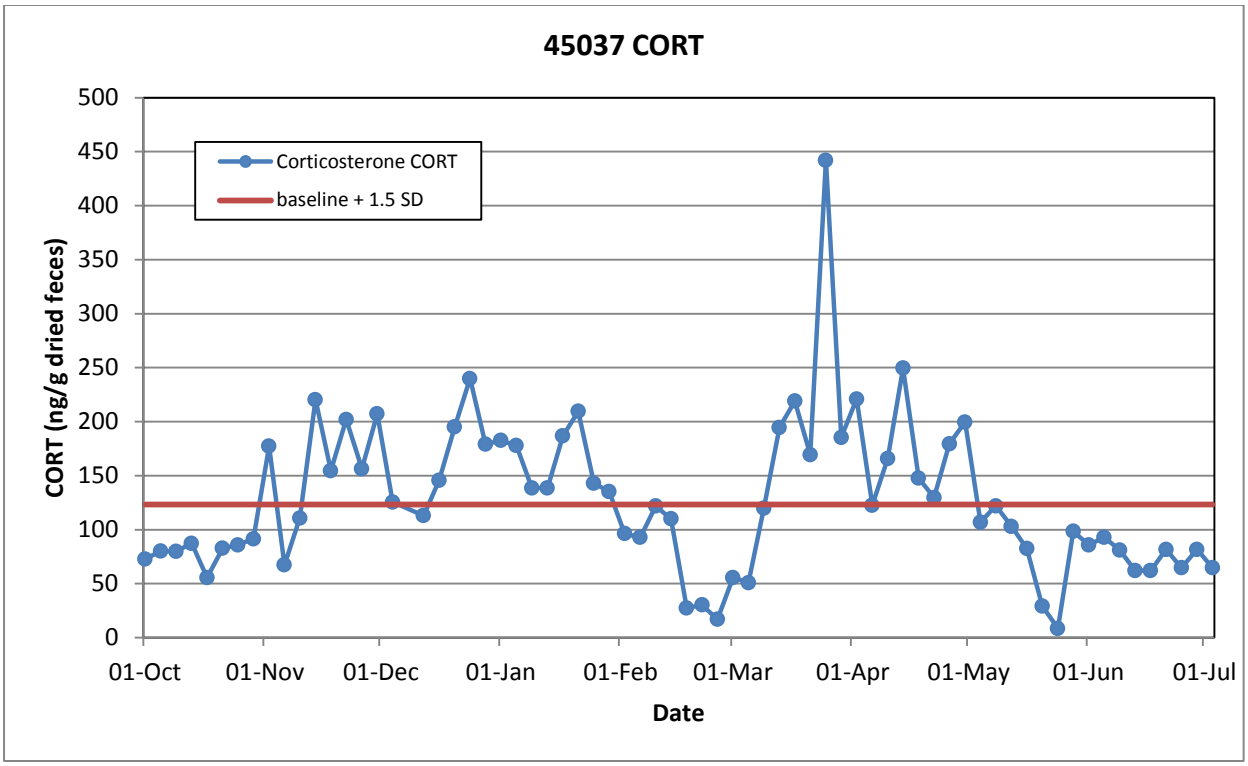

45034 P
Pregnane P
baseline + 1.5 SD
P (ng/g dried feces)
2500
2000
1500
1000
500
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

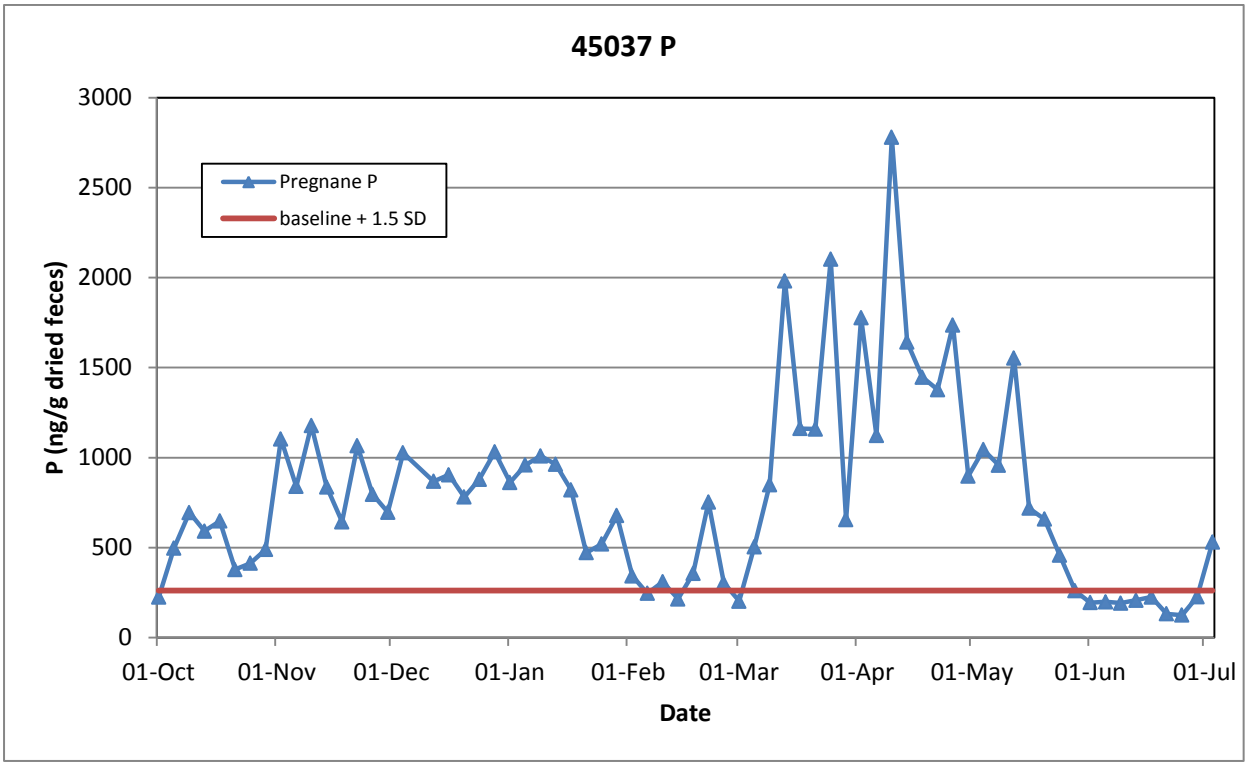

45034 E2
Estradiol E2
baseline + 1.5 SD
E2 (ng/g dried feces)
1800
1600
1400
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

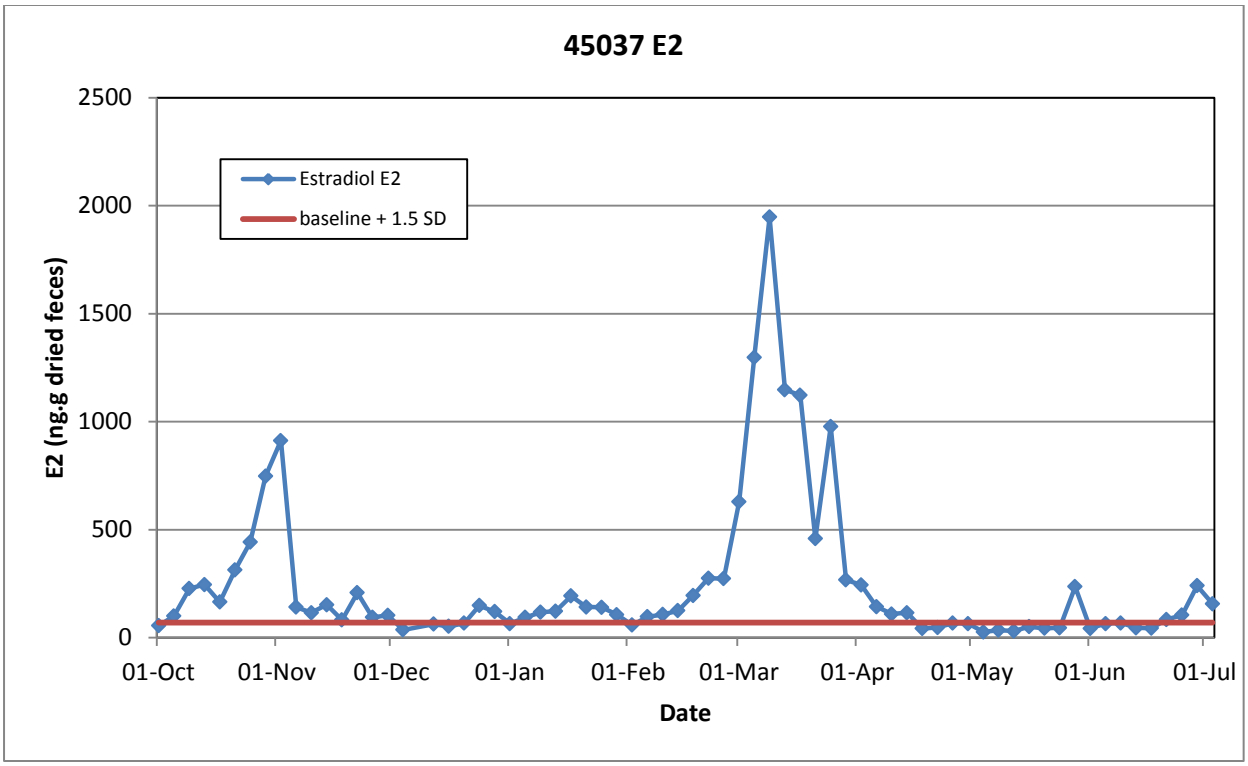

45034 T
Testosterone T
baseline + 1.5 SD
T (ng/g dried feces)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

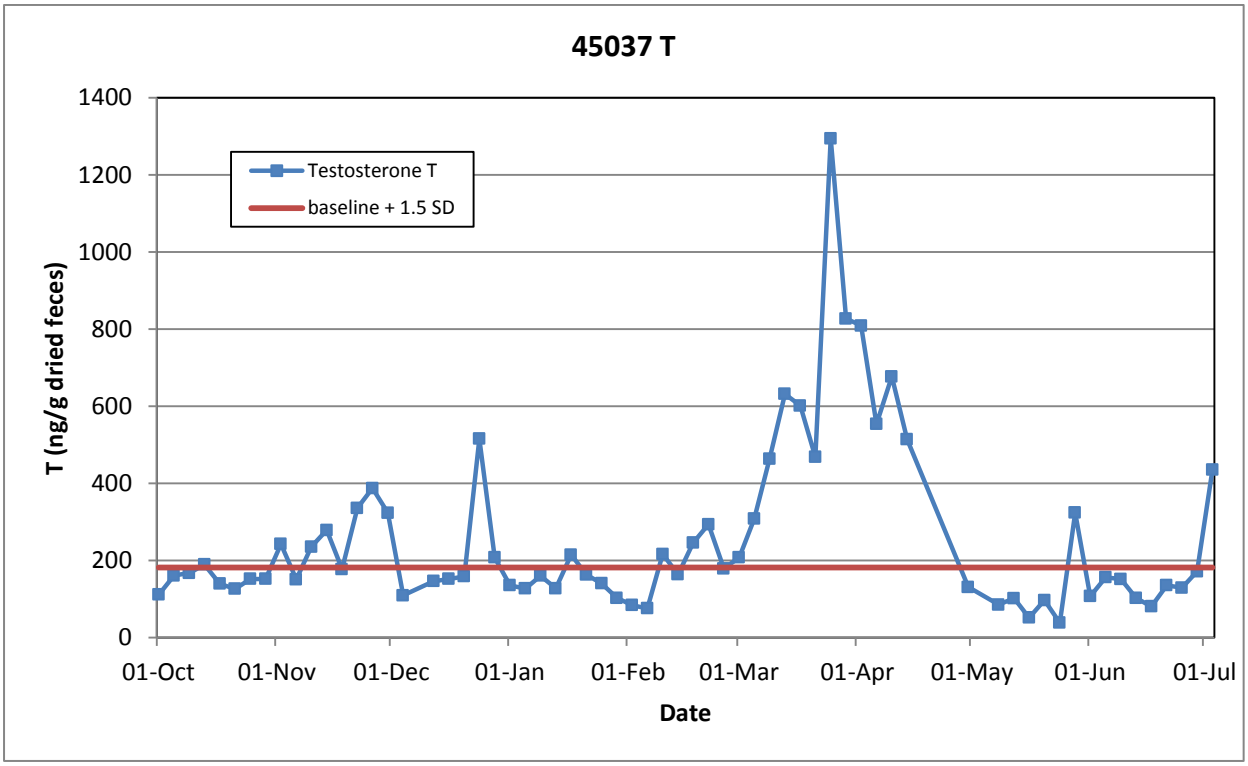

45037
Corticosterone CORT
Tetosterone T
Estrdiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
2500
2000
1500
1000
500
0
12000
10000
8000
6000
4000
2000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
01-Aug
01-Sep
Date

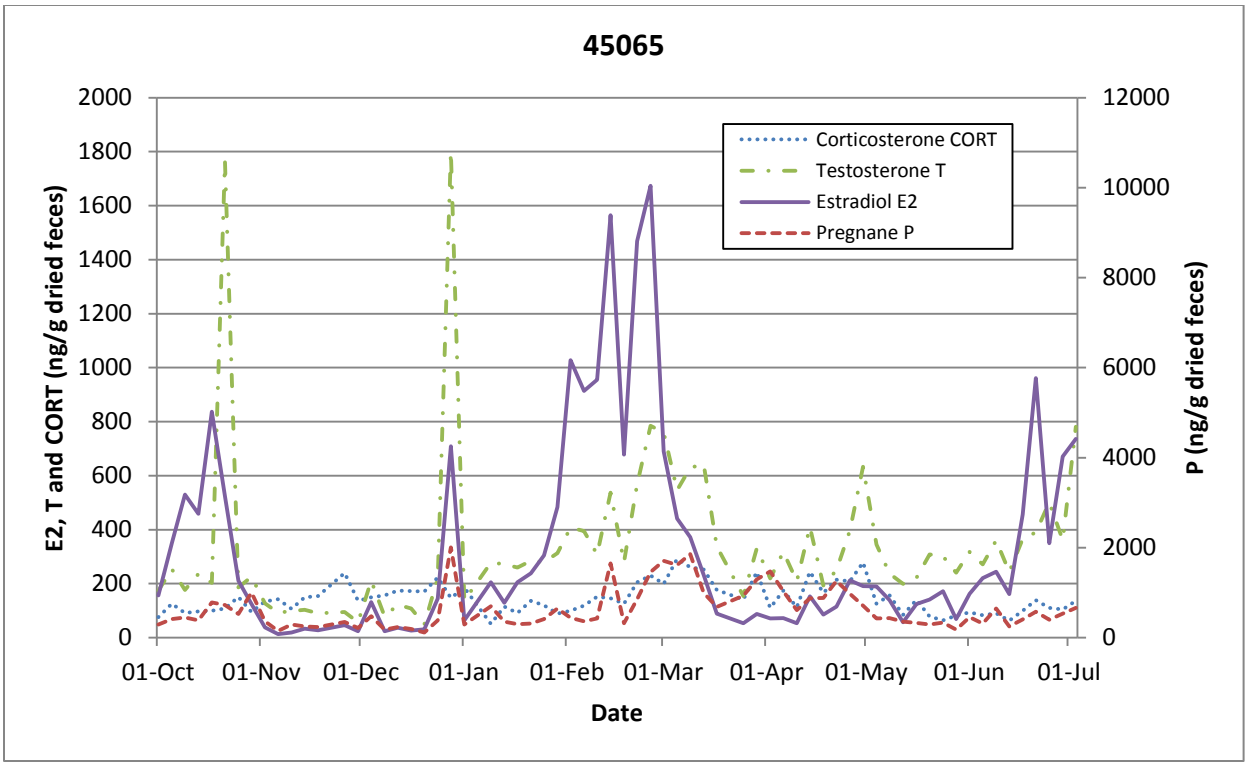

45037 Follicular changes
Length L
Width W
Surface Area SA
L, W (cm) & SA (cm²)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date

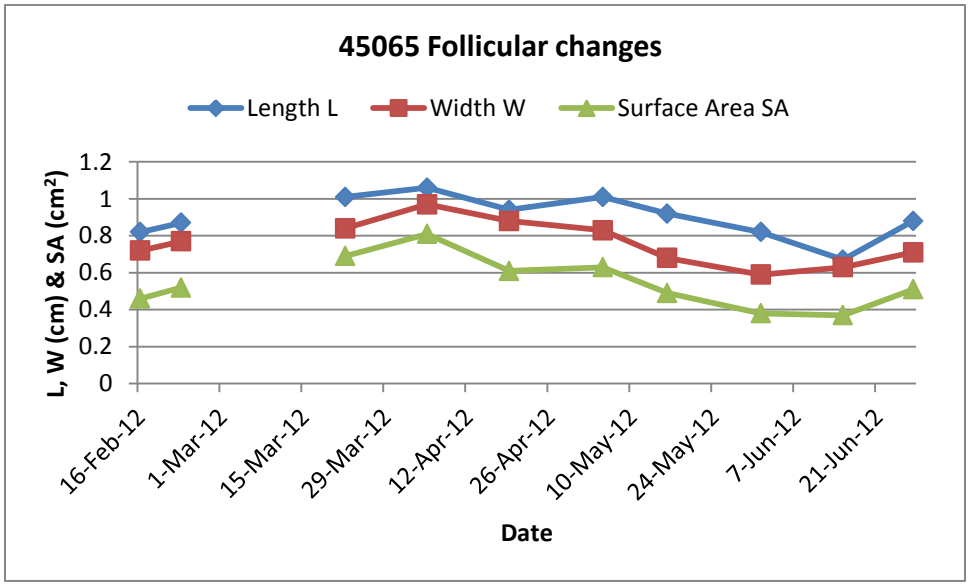

45037 CORT
Corticosterone CORT
baseline + 1.5 SD
CORT (ng/g dried feces)
500
450
400
350
300
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

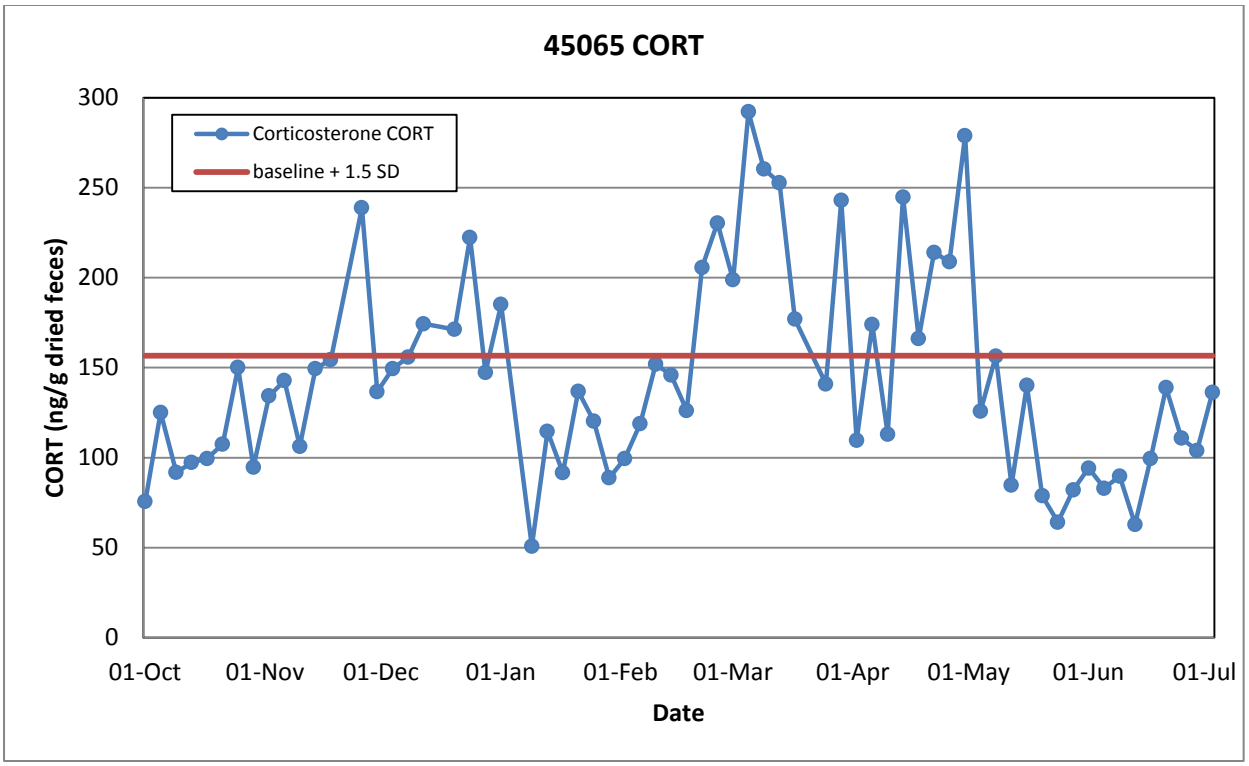

45037 P
Pregnane P
baseline + 1.5 SD
P (ng/g dried feces)
3000
2500
2000
1500
1000
500
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

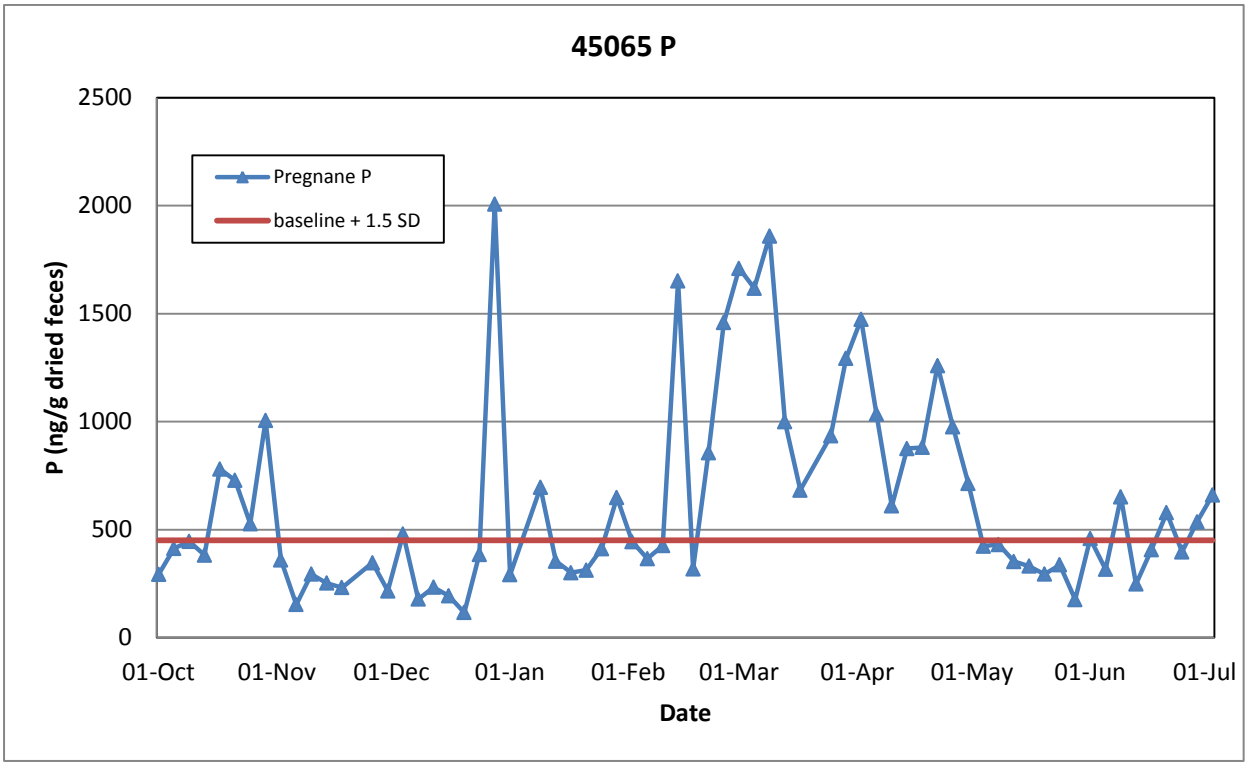

45037 E2
2500
2000
1500
1000
500
0
E2 (ng.g dried feces)
Estradiol E2
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

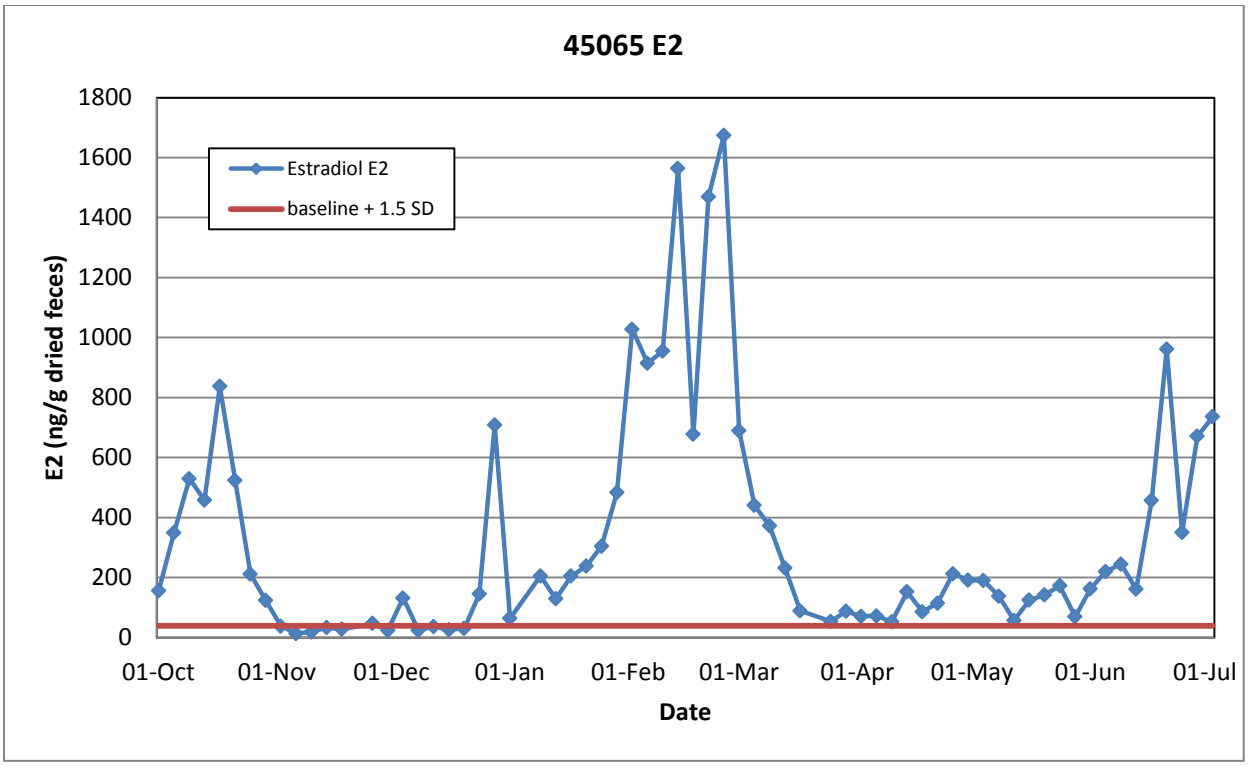

45037 T
1400
1200
1000
800
600
400
200
0
T (ng/g dried feces)
Testosterone T
baseline + 1.5 SD
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

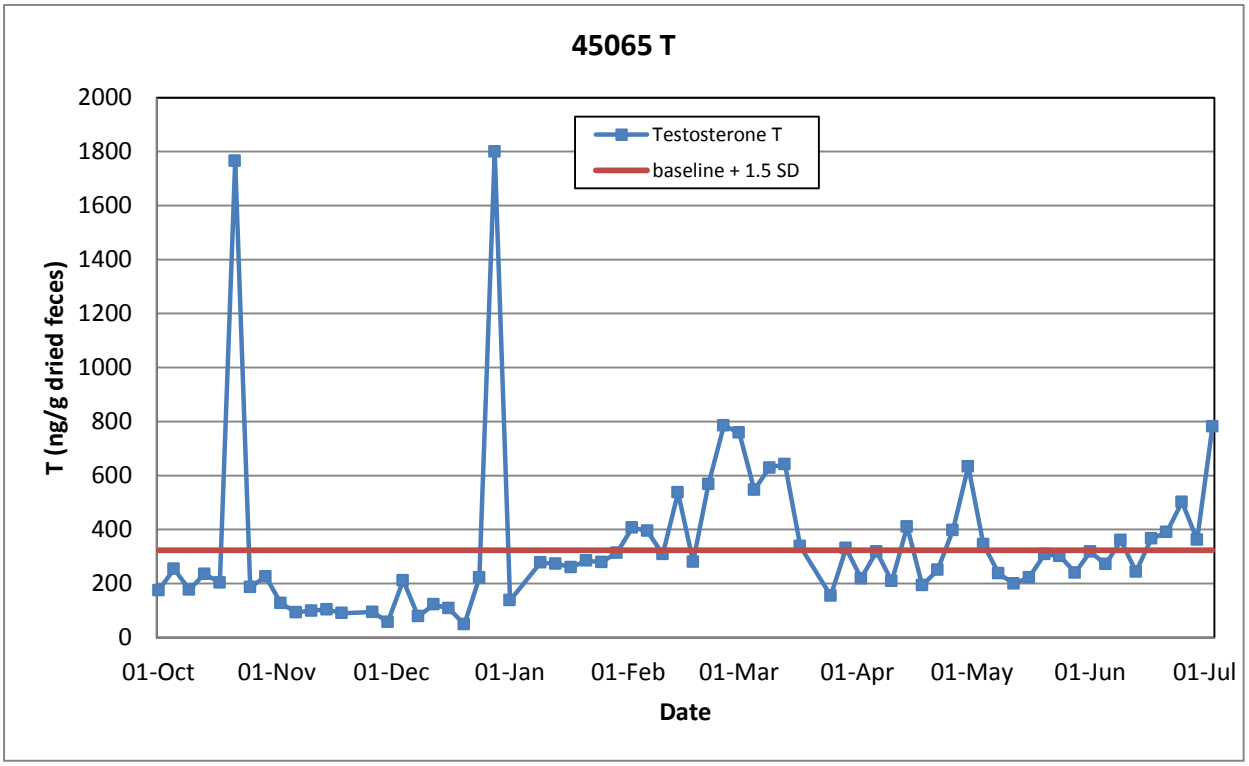

45065
Corticosterone CORT
Testosterone T
Estradiol E2
Pregnane P
E2, T and CORT (ng/g dried feces)
P (ng/g dried feces)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
12000
10000
8000
6000
4000
2000
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date


45065 Follicular changes
Length L
Width W
Surface Area SA
L, W (cm) & SA (cm²)
1.2
1
0.8
0.6
0.4
0.2
0
16-Feb-12
1-Mar-12
15-Mar-12
29-Mar-12
12-Apr-12
26-Apr-12
10-May-12
24-May-12
7-Jun-12
21-Jun-12
Date


45065 CORT
Corticosterone CORT
baseline + 1.5 SD
CORT (ng/g dried feces)
300
250
200
150
100
50
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date


45065 P
Pregnane P
baseline + 1.5 SD
P (ng/g dried feces)
2500
2000
1500
1000
500
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

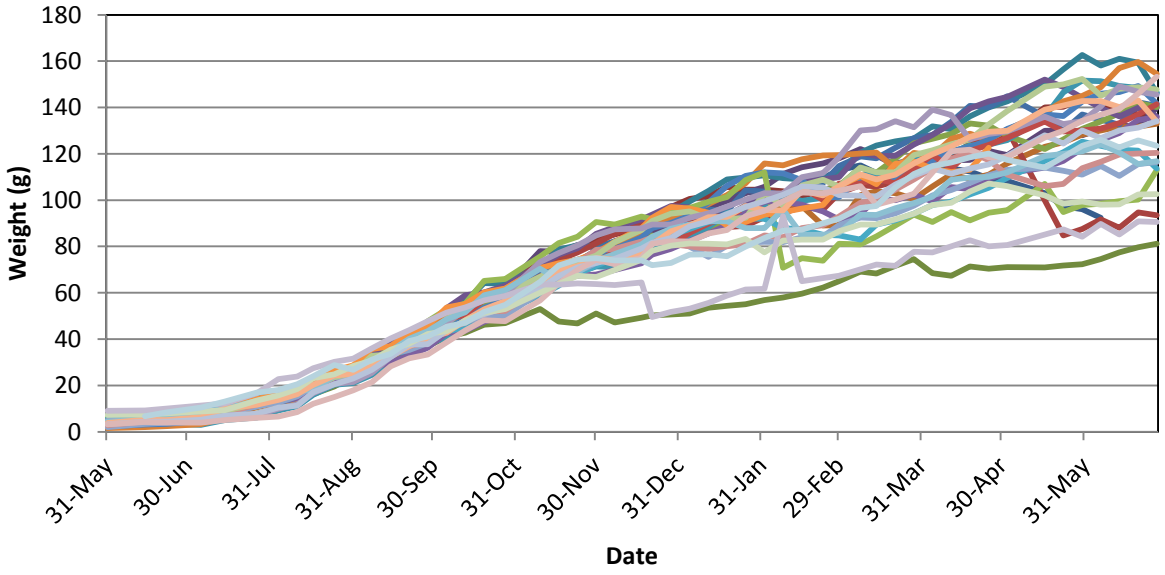

45065 E2
Estradiol E2
baseline + 1.5 SD
E2 (ng/g dried feces)
1800
1600
1400
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date


45065 T
Testosterone T
baseline + 1.5 SD
T (ng/g dried feces)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
01-Oct
01-Nov
01-Dec
01-Jan
01-Feb
01-Mar
01-Apr
01-May
01-Jun
01-Jul
Date

Body weight curves

Body weight (g) of individual chameleons (A) and the mean body weight (g) of the complete study group (B) (n= 28) from the time the animals arrived at Toronto Zoo to the start of the feed restriction trial (May 2011-June 2012).

A



B