Reproductive Physiology of Ethiopian Wolves (*Canis simensis*)

A thesis submitted for the degree of MSc by Research

Freya van Kesteren

Wildlife Conservation Research Unit, Department of Zoology

The Queen's College, University of Oxford

Hillary Term 2011

Abstract

Reproductive physiology of Ethiopian wolves, *Canis simensis*

Freya van Kesteren, The Queen's College MSc by Research, Hillary 2011

Cooperative breeding can be defined as a breeding system in which more than a pair of individuals help rear young from a single litter. Ethiopian wolves, *Canis simensis*, like many other canids, breed cooperatively. However, unlike most group living canids, they specialize in hunting rodents and forage solitarily. Their unusual social system, which combines cooperative breeding, intense sociality and solitary foraging, makes Ethiopian wolves a suitable species in which to study the physiology of cooperative breeding. There are no Ethiopian wolves in captivity, but advances in reproductive science have enabled researchers to study the reproductive physiology of wild populations non-invasively by extracting and assaying hormones in faecal samples using radio/enzyme immunoassays. Using these methods I studied the reproductive physiology of Ethiopian wolves in Bale Mountains National Park, Ethiopia, the largest population of these rare endemic canids.

Faecal samples must be preserved almost immediately after defecation to prevent bacterial degradation of hormones. Freezing is the preferred storage method under controlled conditions, but is often infeasible when studying wild populations. Comparison of three alternative storage methods determined that desiccating samples was a reliable method of preserving Ethiopian wolf samples in field conditions.

Analysis of faecal samples collected from 14 dominant and nine subordinate female Ethiopian wolves revealed that dominant females came into oestrus and showed oestradiol peaks during the annual mating season, whereas subordinate females did not, suggesting a hormonal mechanism of reproductive suppression. However, two subordinate females came into oestrus outside the annual mating season. Both dominant and subordinate females had increased levels of progesterone during the duration of the dominant's pregnancy, and three subordinate females showed signs of pseudopregnancy, including lactation. These results suggest that some subordinate females come into oestrus outside the mating season and become pseudopregnant, as pseudopregnancy in canids follows an infertile ovulation. Dominant females did not prevent subordinate females from mating, and cortisol levels of dominant and subordinate females did not differ significantly during the mating season, suggesting that reproductive suppression in female Ethiopian wolves was unrelated to aggression or stress hormones.

No seasonal patterns in testosterone levels in samples collected from male Ethiopian wolves were found. Subordinate males were observed engaged in mating behaviour, including with their pack's dominant female, but dominant males would prevent subordinate males from mating with the dominant female, suggesting a behavioural method of reproductive suppression. Dominant males had higher cortisol levels than subordinates, which may be related to the stress of maintaining dominant status and/or mate guarding.

In summary, seasonal patterns in progesterone and oestradiol levels were found in female Ethiopian wolves, but not in testosterone levels in males. There was evidence for hormonal and behavioural reproductive suppression of subordinate females and males respectively, but in neither sex was reproductive suppression mediated through stress hormones. As well as providing an insight into the reproductive physiology of a rare cooperatively breeding canid, this study provides needed reproductive biology information for a hitherto unstudied species, and sets the basis for making a contribution to future reproductive management initiatives for the conservation of this charismatic canid.

Acknowledgements

This research could not have been carried out without the support and input of many people, and I am grateful to many colleagues, institutions, friends and family.

Firstly I would like to thank my main supervisor, Dr. Claudio Sillero-Zubiri, and co-supervisors Prof. David Macdonald, Prof. Bob Millar and Dr. Monique Paris for providing me with the opportunity to study the Ethiopian wolves in Bale and for your supervision and guidance. Special thanks go to Claudio for sharing his knowledge of and passion for the wolves with me, to David for his academic input and support, to Bob for help with the laboratory analyses, input and support and to Monique for very helpful discussions and moral support. This research could also not have been carried out without funding provided by the Institute for Breeding Rare and Endangered African Mammals, Dr. Mervyn Jacobson, Queen's College Oxford and the Prins Bernhard Cultuurfonds. Funding for the Ethiopian Wolf Conservation Programme was generously provided by the Wildlife Conservation Network and the Born Free Foundation. I would also like to thank the Ethiopian Wildlife Conservation Authority, and especially Dr. Kifle Agraw, the Oromia Bureau for Agriculture and Rural Development, and the Bale Mountains National Park staff and especially warden Berhanu Jilcha for permission to carry out this research in Bale.

Special thanks go to my field team in Bale; Gedlu Tessera, Muzeyen Turkee, Tamam Mohammed, Yussuf Emeno, Antennah Girma and Hussein Savi for their hard work in collecting the faecal samples and behavioural data. We not only worked side by side for three years but also shared our meals, celebrated holidays and had some great times together. Thanks go to Henrietta Chilton and Stephanie Graham for agreeing to be my volunteers. I am sorry both your visits to Bale were cut short by injuries and wish you better luck in future exploits! Thanks to Melody Kelemu, who volunteered for me only very briefly but became a friend and impressed me with her wisdom at such a young age!

I would like to thank all of the EWCP team, including Anne-Marie Stewart, Chris Gordon, Edriss Ebu, Fikre Getachew and Wegayo Worku for their help and support in Bale. Special thanks go to Anouska Kinnahan and Thadeigh Baggallay from the Frankfurt Zoological Society. I am very grateful for your support and kindness, and for being there for me to turn to when I needed help. Thank you to Charlie Watson and Flavie Vial, the other two Bale firengi gals, both strong, intelligent, and wonderful women. I am happy we shared some experiences in Bale and I'm grateful for your friendship and support. Thanks should also go to the wolves, who became very dear to me and tolerated my endlessly following them and collecting their faecal samples. Individuals such as Orange, White-White, Nafanta and many others enriched my life and if I have made any contribution to the conservation of this species I have accomplished something worthwhile.

At the HRSU in Edinburgh I would like to thank Nancy Evans and Ian Swanston for your help with the lab work, and for your patience and friendliness. Special thanks go to Krissy Nichols, for being another 'poo person', for your help in the lab, and for your friendship. I would like to thank Phil Allcock from Zoology Stores in Oxford for help in sourcing the necessary lab equipment. Thanks go to Prof. Wenche Farstad and Tucker Murphy for comments on earlier drafts, and to Prof. Franz Schwarzenberger for some of the assays.

For the last few years I have been a part of WildCRU, and I have never known such a wonderful, intelligent, dedicated and kind group of people. I found support, acceptance and friendship at WildCRU, and shared stimulating conversations, laughs and good times with everyone here. In particular I would like to thank the following people for their kindness and support: Michelle Lee, Ros Shaw, Alison Poole, Rosie Salazar, Joelene Hughes, Tom Moorhouse, Tom Hart, Merryll Gelling, Sandra Baker, Thomas Merckx, Adam Dutton, Chris Sandom, Christos Astaras, Hanna Tuomisto, Femke Broekhuis, Nic Elliot, Rannveig Magnusdottir, Ruthi Brandt, and Zinta Zommers. Special thanks go to Dawn Burnham and Lynne Larkman for your help and support. Thank you to Lucy Tallents and Jorgelina Marino for helpful discussions about Ethiopian wolves. Special thanks to Erika Cuellar for keeping me company in the paradise! A very special thank you to Paul Johnson. I owe you gratitude not only for help with stats and proof reading, but for your friendship, support and tireless efforts to integrate me into British society through Viz, Carry On, double entendres and cricket. I will miss your humour and wit.

Special bonds develop between office mates at Tubney and I would like to thank my 'original' office mates Joanna Bagniewska and Tucker Murphy. You have shown me what true friendship is and have always been there for me to offer me a shoulder to cry on when I was upset or a dungeon to crash in when I had nowhere to go. You made writing up a lot more tolerable with deep discussions about Lolita and less deep business time songs. I will always be grateful for your love and support and you will both always have a friend in me. But more recent officemates should also not be forgotten. I thank Ewan Macdonald for being a great officemate (who bakes!) and Amy Dickman for great anecdotes and lemons.

There are many friends in Edinburgh whom I'd like to thank, starting with Arno Proeme, an old friend who took me in when I needed a place to live. Thank you to Neelofer Banglawala for your support, positivity, and friendship. Thanks to Kate Sugden and Cosmo Karim for letting me live with you and becoming true friends. Thanks to Stevie Gaffney for girly nights, to Adam Barnett for friendship and late night Google-mapping, to Roger (Lawrence) Mitchell for the best bad movie ever and to Rob Adams for good times and for taking me to the beautiful Lake District.

In Oxford I would like to thank Nicole Milligan, who has been the best friend I could have asked for. Thank you for your unwavering support, friendship, for letting me crash on your floor numerous times over the years and for being the person I could count on when I needed Styrofoam boxes shipped to Ethiopia at short notice. Thank you also to Johanna Frioriksdottir for your friendship, support, sound advice, wonderful chats and good times. Other wonderful friends who supported me during this research include Ewan Mellor, Dominic Ketley, Richard Merrill, Veena Rao, Simon Evans, Chauncy Harris, Sarah Fordham, Ian Preston and Ignacio Silva.

Last but definitely not least I would like to thank my family, who have supported me through this project in so many ways. I could not have done any of this work without the love and support from my mother Ilona Smeets, my father Karel van Kesteren, and my sister Judith van Kesteren. Thank you for everything.

Contents

List of tables

List of Figures

Chapter 1: General Introduction and Methods and Materials

1.1 Vertebrate Reproductive strategies

A range of reproductive strategies exists amongst vertebrates, involving differing degrees of parental investment. In many species parental investment is limited to fertilization and laying of eggs, and neither parent invests in the offspring after hatching (e.g. green sea turtles, *Chelonia mydas*, Hendrickson, 1958). In other species, male investment is limited to fertilization and females care for the young until they become independent (e.g. Kirk's dikdik, *Madoqua kirkii*, Komers, 1996). In yet other vertebrate species both parents invest in raising the young (e.g. barn swallows *Hirundo rustica*, Møller, 1988; California mice, *Peromyscus californicus*, Gubernick & Teferi, 2000). Arguably the most extreme example of investment in young is cooperative breeding, where not only both parents but several helpers invest in young until they become independent (Brown, 1978; Jennions & Macdonald, 1994). This may include providing food for the breeding female, providing food for the young, or guarding young from predators (Brown, 1978). Since evolutionary theory suggests that helpers would benefit more from having their own offspring rather than investing in other's offspring (Cockburn, 1998), researchers have long been intrigued by the biology of cooperative breeders. It is now thought that helpers in cooperatively breeding species are often closely related to the offspring they are helping to rear, and that this may provide evolutionary benefits to helpers who are environmentally constrained from having their own offspring (for a review see Jennions & Macdonald, 1994).

Cooperative breeding has been described in fish such as the daffodil cichlid, *Neolamprologus pulcher* (Balshine-Earn et al., 1998) , many bird species including Florida scrub jays, *Aphelocoma coerulescens* and white-winged choughs, *Corcorax melanorhamphos* (Brown, 1978), and mammals including common marmosets *Callithrix jacchus* (Barrett et al., 1990), meerkats, *Suricata suricatta* (Doolan & Macdonald, 1999),

and African wild dogs, *Lycaon pictus* (Creel et al., 1997a). Cooperative breeding requires the reproductive suppression of subordinates, and, as reproduction is linked to hormones such as oestradiol and progesterone in females and testosterone in males (Hadley, 2000), a hormonal mechanism for reproductive suppression has been sought and found in a variety of species such as female common marmosets (Barrett et al., 1990) and Damaraland mole rats, *Cryptomys damarensis* (Bennett et al., 1996). As well as hormonal mechanisms of reproductive suppression, there are behavioural mechanisms, which involve dominants preventing subordinates from mating (e.g. male dwarf mongooses *Helogale parvula*, Creel et al., 1992). Ethiopian wolves, *Canis simensis,* are cooperative breeders, and usually only the dominant female in a pack breeds, with one or more subordinate females forgoing reproduction whilst remaining in their natal pack (Sillero-Zubiri et al., 1996a; Sillero-Zubiri et al., 2004b). Within a pack the dominant female mates only with the dominant male, but extra-pack copulations are common (Sillero-Zubiri et al., 1996a) and extra-pack paternity has been found in litters (Randall et al., 2007; Gottelli et al., 1994). However, although some aspects of Ethiopian wolf reproduction have been studied before using behavioural (Marino et al., 2006; Sillero-Zubiri & Gottelli, 1995a; Sillero-Zubiri & Gottelli, 1995b; Sillero-Zubiri et al., 1996a; Sillero-Zubiri et al., 1998; Sillero-Zubiri & Macdonald, 1998) and genetic methodologies (Gottelli et al., 1994; Randall et al., 2007) their reproductive biology and the mechanism by which subordinates are reproductively suppressed are poorly understood.

1.2 Why study the sex life of the world's rarest canid?

Ethiopian wolves diverged from a wolf-like ancestor that colonized the African continent about 100,000 years ago, and evolved into a species that has adapted to its highland environment (Gottelli et al., 2004). Ethiopian wolves specialize in hunting the rodents that

live in their habitat, and in the Bale Mountains, the endemic giant molerat, *Tachyoryctes macrocephalus*, is the wolves' main prey (Sillero-Zubiri & Gottelli, 1995a). Because Ethiopian wolves specialize in hunting small rodents, they usually forage solitarily, although on occasion groups of wolves may hunt Starck's hares, *Lepus starcki*, young antelopes or sheep (Sillero-Zubiri & Gottelli, 1995a). The fact that Ethiopian wolves forage solitarily and yet often live in packs make them somewhat unusual, since other pack-living canids including African wild dogs (Creel & Creel, 1995), and grey wolves, *Canis lupus* (Thurber & Peterson, 1993), hunt cooperatively.

Ethiopian wolf dispersal is thought to be constrained by the availability of suitable habitat, and packs form, and grow chiefly as a consequence of male philopatry. Larger packs with more males secure larger territories, with better food resources (Marino et al., submitted) and have larger litters at emergence (Tallents, 2007). Within packs, however, only the dominant pair breed and the remaining pack members help rear the pups, for example through den-guarding, provisioning prey to the pups, or allosuckling (Sillero-Zubiri et al., 1996a; Sillero-Zubiri et al., 2004b). This breeding system, however, carries a potential risk of inbreeding. Ethiopian wolves seem to avoid inbreeding through female-biased dispersal and mating behaviour, and as a result breeding pairs are typically unrelated (Randall et al., 2007). Although within a pack only the dominant male mates with the dominant female, both dominant and subordinate males participate in extra-pack copulations (Sillero-Zubiri et al., 1996a), and subordinate males may sire pups (Randall et al., 2007). Subordinate females can choose between different strategies: they may stay in their natal pack and help rear related pups, and perhaps one day replace their mother as the dominant, breeding female; they may disperse in the hope of finding a breeding opportunity in a different pack (Sillero-Zubiri et al., 1996a); or they may try to split an existing pack (pack fission) and

become the dominant female in the new pack (Marino, 2003a). Although each of these strategies has been observed in the wild, nothing is known about the physiological mechanisms underlying female Ethiopian wolf reproductive behaviour.

Less than 500 adult Ethiopian wolves survive in a handful of highland enclaves in Ethiopia (Marino, 2003b), giving this species the dubious honour of being the world's rarest canid (Marino et al., 2006). Threats to Ethiopian wolf survival include habitat destruction and fragmentation (Sillero-Zubiri & Macdonald, 1997), but the most immediate threat is disease spread by domestic dogs, notably rabies. Rabies outbreaks greatly reduced wolf populations in the Bale Mountains in 1991-92 (Sillero-Zubiri et al., 1996b), 2003 (Haydon et al., 2006) and most recently in 2008-09 (Johnson et al., 2010, see Appendix).

The Ethiopian wolf is a large and charismatic mammal, and its conservation is important for several reasons. Ethiopian wolves act as a flagship species for their Afroalpine ecosystem (Sillero-Zubiri & Macdonald, 1997), which is home to many other rare and endemic species including, among others, the mountain nyala *Tragelaphus buxtoni*, giant molerat, and Starck's hare (Yalden & Largen, 1992). The Bale Mountains National Park, home to the largest remaining population of Ethiopian wolves (Sillero-Zubiri & Gottelli, 1994), has such a high proportion of endemic species that with the loss of the Park more species would become extinct than with any other area of comparable size worldwide (FZS, 2010). In addition, the Bale Mountains are an important water catchment area for regions in both Ethiopia and Somalia (Hillman, 1988). Ethiopian wolves are also the Park's chief tourist attraction (Macdonald & Sillero-Zubiri, 2004), and tourism brings much needed business and revenue to the area.

In order to conserve any species, it is imperative to understand the species' biology. The behavioural ecology of Ethiopian wolves has been studied extensively and aspects of Ethiopian wolf biology, including diet (Sillero-Zubiri & Gottelli, 1995a), territoriality (Sillero-Zubiri & Macdonald, 1998), and breeding system (Sillero-Zubiri et al., 1996a) are now well understood. Aspects of Ethiopian wolf reproduction have been studied previously using behavioural observations and molecular genetics. These include mating behaviour (Sillero-Zubiri et al., 1996a), occurrence of breeding seasonality and synchronicity (Sillero-Zubiri et al., 1998), pack dynamics (Marino et al., 2006) determinants of reproductive success (Tallents, 2007) and inbreeding avoidance (Randall et al., 2007). However, nothing is known about the reproductive physiology of Ethiopian wolves. Metapopulation management, sperm and egg cell banking and captive breeding have all been recommended as possible tools for the conservation of Ethiopian wolves (Sillero-Zubiri et al., 2004a; Sillero-Zubiri & Macdonald, 1997). A greater understanding of the reproductive physiology of Ethiopian wolves is arguably an important research priority for this species and would form the basis of future initiatives of reproductive management. Although there are no Ethiopian wolves in captivity (Sillero-Zubiri & Macdonald, 1997), due to technical advances in non-invasive reproductive physiology techniques, it is now possible to study wild populations through the collection of faecal or urine samples (e.g. Schwarzenberger, 2007; Wildt & Wemmer, 1999). These technical advances have now made it possible to study the reproductive physiology of Ethiopian wolves in the wild.

The interesting breeding and social system of Ethiopian wolves, their status as the world's rarest canid, as well as the fact that there are no Ethiopian wolves in captivity make this an ideal species for a non-invasive study of reproductive physiology. This thesis investigates seasonal patterns in oestradiol and progesterone in female Ethiopian wolves, and

testosterone in male Ethiopian wolves. The mechanisms of reproductive suppression in male and female subordinate wolves are studied, both through behavioural observations and through faecal hormone assays. Furthermore, the relationship between dominance status and cortisol levels is investigated. This study represents the first effort to assess the reproductive physiology of Ethiopian wolves, and provides new understanding of the reproduction of this rare canid.

1.3 Research aims and structure of this thesis

This thesis aims to describe, for the first time, the reproductive physiology of Ethiopian wolves. More specifically, it tests hypotheses regarding seasonal patterns in reproductive hormones in both male and female Ethiopian wolves, as well as to the mechanism of reproductive suppression in subordinate wolves. It also tests hypotheses on the relationship between dominance status, aggression, reproduction and cortisol. Although some aspects of Ethiopian wolf reproduction have been studied before (Sillero-Zubiri et al., 1996a; Sillero-Zubiri et al., 1998; Tallents, 2007; Marino et al., 2006; Randall et al., 2007), nothing is known about the reproductive physiology of this species. As well as this chapter (a general introduction and methods and materials section), this thesis consists of a review of canid reproductive physiology (Chapter 2), three data chapters (Chapters 3, 4 and 5), and a general discussion (Chapter 6). The outline of this thesis is as following:

- **Chapter 2: Every dog has its way: a review of canid reproductive physiology**

  The reproductive physiology of domestic dogs and several wild canid species has been studied in detail. Several reproductive features, including cooperative breeding, reproductive suppression of subordinates, and alloparental care have been found in different canid species (Asa & Valdespino, 1998), and the reproductive phases described in domestic bitches (Jöchle & Andersen, 1976), have also been described in species that are genetically closely related to Ethiopian wolves

including grey wolves (Seal et al., 1979) and coyotes, *Canis latrans* (Carlson & Gese, 2008). Reproductive physiology of males has also been studied in several species including domestic dogs (Ortega-Pacheco et al., 2006), grey wolves (Kreeger, 2003), coyotes (Minter & DeLiberti, 2008), and African wild dogs (Johnston et al., 2007). A literature review on the current state of knowledge of the reproductive physiology of canid species was conducted to provide a background to the subsequent chapters of this thesis.

- **Chapter 3: Preservation of Ethiopian wolf faecal samples for hormone analysis**

  Bacterial degradation of hormones in faecal samples starts almost immediately after defecation (Wasser et al., 1988), so samples must be preserved as soon as possible. The preferred method of preserving faecal samples is to freeze them (e.g. Terio et al., 2002), but this is often infeasible when studying wild populations in remote locations. Several alternative storage methods have been described in the literature, including preserving samples in ethanol (e.g. Wasser et al., 1991) and desiccating samples (e.g. Brockman & Whitten, 1996). However, since different storage methods can yield different results for individual species and hormones studied, several authors recommend validating alternative storage methods for each new species studied (Buchanan & Goldsmith, 2004; Khan et al., 2002; Terio et al., 2002). In this study, freezing samples in the field proved to be very difficult due to logistical problems, and therefore finding an alternative sample storage method was essential. The aim of this chapter was therefore to find and validate a more practical method of preserving Ethiopian wolf faecal samples for hormone analysis.

- **Chapter 4: Sex, suppression and pseudopregnancy in female Ethiopian wolves**

As Ethiopian wolves are seasonal breeders (Sillero-Zubiri et al., 1998), one aim of this thesis was to study whether there are seasonal patterns in females' reproductive hormones, namely oestradiol and progesterone. We expected that dominant female Ethiopian wolves would show an oestradiol surge (associated with oestrus) only once a year, during the annual mating season. Pregnant females would show higher levels of progesterone during pregnancy. Subordinate female Ethiopian wolves generally do not mate or breed (Sillero-Zubiri et al., 1996a, Chapter 4), but may allosuckle the dominant female's pups (Sillero-Zubiri et al., 1996, Chapter 4) suggesting that they may become pseudopregnant (Creel et al., 1991). Pseudopregnancy in other canids follows an infertile oestrus, (e.g. Chakraborty, 1987) which suggests that at least some subordinate females do come into oestrus. We therefore predicted that some subordinate females would come into oestrus and become pseudopregnant, and therefore also show increased levels of progesterone during the time that their dominant female is pregnant. Lastly, as cortisol may play a role in reproductive suppression (see Creel, 2001), we studied patterns in cortisol levels between dominant and subordinate females, and related cortisol levels with rates of aggression between females. Higher cortisol levels have been found in dominant females of several canids including grey wolves (Sands & Creel, 2004) and African wild dogs (Creel et al., 1997a), so we expected dominant females to have higher cortisol levels than subordinate females.

- **Chapter 5: Sex, stress and social status: patterns in testosterone and cortisol in male Ethiopian wolves**

  Most canids including coyotes (Minter & DeLiberti, 2008) and grey wolves (Seal et al., 1979) are seasonal breeders, with one breeding season per year. In some species studied, such as Arctic foxes, *Alopex lagopus* (Smith et al., 1985) and coyotes

(Minter & DeLiberti, 2008), testosterone follows a seasonal pattern, peaking during the mating season. Differences in both testosterone and cortisol levels between dominants and subordinates have also been found in some canid species, including African wild dogs (Creel et al., 1997a; Johnston et al., 2007). One theory which predicts seasonal patterns in testosterone levels is the Challenge Hypothesis (Wingfield et al., 1990), which posits that testosterone levels should rise from baseline levels to slightly higher levels during the mating season. Further increases in testosterone are expected to be related to aggression rather than reproduction. Males in species that show paternal care should show lower levels of testosterone when there are young to care for than males in species which do not show paternal care. Although the Challenge Hypothesis is based largely on data from birds, several mammals have been found to conform to it, including chimpanzees *Pan troglodytes schweinfuhrtii* (Muller & Wrangham, 2004) and degus, *Octoden degus* (Soto-Gamboa et al., 2005). We would expect Ethiopian wolves to show higher testosterone levels during the mating season, when aggression levels are also highest, and lower levels when there are pups to care for. However, since both dominant and subordinate males may mate and sire pups (Randall et al., 2007; Sillero-Zubiri et al., 1996a), we would not expect to see significant differences in testosterone levels of dominant and subordinate males. We would expect dominant males to have higher cortisol levels than subordinate males, as this has been found in other canid species including African wild dogs and grey wolves (Creel et al., 1997a; Johnston et al., 2007; Sands & Creel, 2004).

- **Chapter 6: General discussion**

This chapter summarizes the results from chapters three, four and five, and discusses these results in the wider context of Ethiopian wolf conservation. The limitations of non-invasive reproductive physiology studies are also discussed. The results from this study are related to Ethiopian wolf conservation efforts associated with reproductive management, and suggestions are made for future reproductive management initiatives.

1.4 Ethiopian wolves: biology and behaviour

Ethiopian wolves are medium sized territorial canids endemic to the highlands of Ethiopia, with more than half the remaining population occurring in Southern Ethiopia's Bale Mountains (Sillero-Zubiri & Gottelli, 1994). Ethiopian wolves live in family packs consisting of 2-13 adult wolves, 0-6 yearlings and 0-6 pups (Sillero-Zubiri et al., 1996a). Packs occupy and defend territories, which are on average about 6 km^2 in size (Sillero-Zubiri & Gottelli, 1995b). All adult members of a pack participate in daily territory boundary patrols, mark territory boundaries with scent marks, and defend their pack territory from wolves in neighbouring packs (Sillero-Zubiri & Macdonald, 1998). Although Ethiopian wolves live in packs, they mainly forage solitarily and specialize in hunting Afroalpine rodents, especially the endemic giant molerat, the Ethiopian wolf's main food item in the Bale Mountains (Sillero-Zubiri & Gottelli, 1995a), where this study was based.

Ethiopian wolves are cooperative breeders, and generally only the dominant female in a pack breeds (Sillero-Zubiri et al., 1996a). All members of the pack participate in rearing the dominant female's pups, which includes den guarding and regurgitating prey to the pups. Subordinate females may also allosuckle the pups (Sillero-Zubiri et al., 1996a). Within a pack, the dominant female mates with only the dominant male. However,

Sillero-Zubiri et al. (1996a) found that 70% of the 30 copulations observed were between the dominant female of one pack and a male (either dominant or subordinate) from a neighbouring pack. Furthermore Randall et al. (2007a) found that extra-pack paternity accounted for 28% of all resolved paternities, and occurred in half of the twelve litters studied.

Ethiopian wolves breed seasonally, and pups are usually born at the end of the rainy season (October-January). Gestation lasts about 60 days and litters usually consist of 1-6 pups (Sillero-Zubiri & Gottelli, 1994). Within subpopulations, packs show breeding synchrony, with all dominant females giving birth within a 1-5 week period. This breeding synchrony is thought to benefit breeding females by increasing the dominant male's paternal investment in her young (Sillero-Zubiri et al., 1998). Male Ethiopian wolves tend to be philopatric and remain in their natal pack, whilst females are more likely to disperse (Sillero-Zubiri et al., 1996a). Dispersal opportunities are thought to be limited by habitat saturation (Sillero-Zubiri et al., 1998). Males and females reach sexual maturity in their second year (Sillero-Zubiri & Gottelli, 1994). Genetic studies have shown that Ethiopian wolves are more closely related to grey wolves and coyotes than to any other African canid. Ethiopian wolves are also closely related to domestic dogs (*Canis familiaris*), a recent derivative of the grey wolf, and may hybridize with domestic dogs (Gottelli et al., 1994).

1.5 Cooperative Breeding and Reproductive Suppression

Cooperative breeding can be defined as a breeding system in which more than a pair of individuals exhibit helping behaviour towards young from a single litter, or where individuals assist the breeding pair (Jennions & Macdonald, 1994). Cooperative breeding

involves three main attributes, namely delayed dispersal from the natal group, reproductive suppression and care for others' offspring (Solomon & French, 1997). In most cooperatively breeding mammals, reproductive rates are lower for subordinates than for dominants, and it is common for reproduction in subordinates to be completely suppressed (Creel, 2001). Reproductive suppression may occur when ecological and demographic conditions limit breeding opportunities, constraining the reproductive options of young, competitively inferior individuals (Creel & Creel, 1991). Reproductive suppression is also thought to be related to the energy costs of breeding. Subordinate females of species in which the energetic costs of gestation and lactation are high should be more likely to tolerate reproductive suppression. In communally breeding species the costs of reproduction may be so high that a single breeding pair cannot successfully rear young (Creel & Creel, 1991). This appears to be the case in Ethiopian wolves, as all pack members contribute to the rearing of the pups (Sillero-Zubiri et al., 1996a), and single breeding pairs do not usually succeed in rearing pups. Only one case of a single pair breeding successfully was recorded by the Ethiopian Wolf Conservation Programme (EWCP) in six years (Stewart et al., 2010). In this case, only one pup emerged from the breeding pair's den.

Reproductive suppression may occur through various mechanisms, which can be divided into hormonal and behavioural mechanisms. One hormonal mechanism by which subordinates are thought to be reproductively suppressed is through increased glucocorticoids, a class of steroid hormones produced in the adrenal gland. Glucocorticoids are released in response to adverse situations, and as such are seen as indicators of stress (Möstl & Palme, 2002), and often referred to as 'stress hormones'. The type of glucocorticoid most prevalent differs amongst species. In rodents the most common

glucocorticoid is corticosterone, whereas in medium and large mammals, including humans, cortisol is the most common glucocorticoid (Millspaugh & Washburn, 2004).

Short term elevation of glucocorticoids may lead to adaptive behavioural and physiological processes to deal with stress, but chronic elevation of glucocorticoids may cause reproductive failure and disease (Goymann et al., 2001; Chrousos & Gold, 1992). Cortisol has been shown to delay or prevent the oestradiol induced luteinizing hormone (LH) surge required for ovulation in domestic sheep (Pierce et al., 2009), and chronic elevation of glucocorticoids can produce a decrease in testosterone secretion through hypothalamic-pituitary-gonadal (HPG) axis inhibition, thus lowering an individual's testosterone levels (Blanchard et al., 2001).

In group-living vertebrates which have dominance hierarchies, differences in glucocorticoid levels are often found (Creel, 2001). Early studies on dominance rank and stress were done in captive situations, using rodents and primates (see Creel, 2001; for an example see Louch & Higginbotham, 1967). In these studies two individuals were put together and aggressive interactions ensued. Glucocorticoids were then measured in both the winner and the loser of the interaction. In this situation, both winners and losers had increased levels of glucocorticoids, but the stress response was higher in losers, leading to the 'stress of subordination' theory (see Creel, 2001). Some studies in wild populations did find that subordinates had higher glucocorticoid levels, for example non-lactating female spotted hyaenas, *Crocuta crocuta* (Goymann et al., 2001) and alpine marmots, *Marmota marmota* (Arnold & Dittami, 1997). These studies support the hypothesis that in some species subordinate animals may be reproductively suppressed through stress hormones.

More recently, however, it has been suggested that aggression is stressful to both the instigator and the recipient (e.g. Creel et al., 1996). In the wild, subordinate individuals may move away from dominant individuals to avoid aggression, but dominants may have to act aggressively more often to maintain their dominant status, and this may be stressful. According to this theory, increased glucocorticoid levels are a cost of dominance rather than a consequence of subordinance, and this is known as the 'stress of dominance' theory (Creel, 2001). In support of this theory, several studies have found a link between aggression and cortisol. For example, aggressive domestic dogs show higher plasma cortisol levels than non-aggressive dogs (Rosado et al., 2010), and aggression and cortisol levels are related in several species (e.g. ring-tailed lemurs, *Lemur catta*, Cavigelli et al., 2003).

Creel (2001) reviewed 25 studies of glucocorticoid levels in cooperative and non-cooperative breeders. Eight of these studies found that dominants had higher glucocorticoid levels, eight found that subordinates had higher glucocorticoid levels, and eight found no significant difference. All studies showing higher glucocorticoid levels in dominants involved cooperative breeders (Creel, 2001). Limited data from African wild dogs and dwarf mongooses show that glucocorticoid levels increase upon attaining dominance (Creel et al., 1996) and both male and female dominant African wild dogs (Creel et al., 1997a) and dwarf mongooses (Creel et al., 1996) had higher glucocorticoid levels than subordinates. Similarly, Sands and Creel (2004) found that faecal glucocorticoid levels were significantly higher in dominant grey wolves of both sexes than in subordinates.

Dominant females may also show higher glucocorticoid levels than subordinates for reasons unrelated to aggression. For example, increased glucocorticoids have been linked to reproductive events such as ovulation, pregnancy, and parturition (e.g. Cavigelli, 1999; Saltzman et al., 1998) and in domestic cats (*Felis catus*, Finkler & Terkel, 2010) and common marmosets (Saltzman et al., 1998) ovariectomy was found to reduce cortisol levels. In species in which only the dominant female breeds (such as Ethiopian wolves), only the dominant female would experience these reproductive events thus potentially leading to higher glucocorticoid levels than those of subordinate, non-breeding females. However, studies in species which have dominance hierarchies but in which subordinate females also breed (e.g. ring tailed lemurs, Cavigelli et al., 2003), show that increased glucocorticoids in dominants are not solely attributed to reproductive stresses. Cavigelli et al. (2003) suggested several other reasons that dominants may show higher GC levels including increased aggression, higher sensitivity of the hypothalamic-pituitary-adrenal (HPA) axis, which controls stress response, and even a link between fat stores and glucocorticoids, a relationship that has not yet been well studied.

Subordinates can also be reproductively suppressed through other hormones. For example, subordinate female Damaraland molerats had reduced basal levels of luteinizing hormone (LH) and elevated progesterone levels when in the presence of a dominant, breeding female (Bennett et al., 1996). The combination of low LH and high progesterone prevents ovulation, but removal of the dominant female resulted in increased LH and decreased progesterone, proving that reproductive suppression can be reversed in this species (Bennett et al., 1996). Similarly, female common marmosets showed low levels of LH in the presence of a dominant female, preventing ovulation. Removal of the dominant female prompted an increase in plasma LH and the onset of ovulation, although ovulation was

delayed if subordinates were exposed to scent cues from their dominant female, suggesting reproductive suppression in marmosets is mediated through pheromones (Barrett et al., 1990). Similarly, Creel et al. (1997a) found that subordinate female African wild dogs had lower oestrogen levels than did dominants during the mating season.

Reproductive suppression may also be achieved through behavioural mechanisms, as opposed to hormonal mechanisms. In captivity, dominant male grey wolves prevented subordinates from mating through aggressive behaviours, especially when a subordinate tried to mate with the dominant's preferred female (Derix et al., 1993). Creel et al. (1992) found that, whilst subordinate female dwarf mongooses were hormonally suppressed and had lower oestrogen levels, subordinate males were primarily suppressed through behavioural mechanisms. Subordinate males were found to have testosterone concentrations indistinguishable from dominants, but were prevented from mating with the dominant female through aggressive behaviour by the dominant male. Similarly, although female Damaraland molerats were hormonally reproductively suppressed, subordinate males had similar testosterone levels as dominants, and can produce sperm, but were prevented from mating with the dominant female (Bennett, 2009). This research on dwarf mongooses (Creel et al., 1992) and Damaraland molerats (Bennett, 2009) shows that both behavioural and endocrine mechanisms can play a role in reproductive suppression within one species.

1.6 Faecal Hormone Analysis

Endocrine glands secrete hormones directly into the circulatory system (Hadley, 2000), and hormone levels can be measured in plasma and serum samples (e.g. Concannon et al., 1978). However, collecting blood samples is invasive as it requires restraining and handling an individual. Once hormones have delivered their message they are broken down

in the liver and excreted in faeces or urine (Hadley, 2000). Recently, innovative techniques have been developed to measure hormones in faecal or urine samples (Whitten et al., 1998), creating new possibilities for empirical studies on reproductive physiology of wild populations.

Faecal hormone analysis has been used by researchers to study reproduction and/or stress in several species. Excreted samples are very useful in the study of free ranging animals because it is possible to regularly collect samples for hormone evaluations without darting or trapping (Fujita et al., 2001). Initially, studies on faecal (or urinary) hormones were done only with captive animals in zoos, such as red buffalo, *Syncerus caffer nanus*, yak, *Bos mutus*, Grevy's zebra, *Equus grevy*, Nubian ibex, *Capra ibex nubiana* and hippopotamus *Hippopotamus amphibious* (Safar-Hermann et al., 1987) or in domesticated animals such as horses, *Equus caballus* (Bamberg & Schwarzenberger, 1990). More recently, however, there have been numerous studies in wild populations, including populations of black rhinoceros *Diceros bicornis minor* (Garnier et al., 2002), yellow baboons, *Papio cynocephalus* (Wasser, 1996), dwarf mongooses (Creel et al., 1992), and African wild dogs (Creel et al., 1996).

Hormones are metabolised and excreted differently in different species, and ideally, the pathway of hormone excretion is determined for each study animal. One way of testing how hormones are excreted by different species is to inject a study animal with a radio-labelled hormone, and testing for excretion of that hormone in faeces and urine. For example, Velloso et al. (1998) injected one male maned wolf, *Chrysocyon brachyurus*, with radio-labelled testosterone and found that about 97% of metabolized testosterone was excreted through faeces. Similarly, Montfort et al. (1997) injected one female African wild

dog with radio-labelled oestradiol and progesterone, and found that these were excreted in equivalent portions in urine and faeces. In some cases, a model organism is used to study how hormone metabolites are excreted. For example, Brown et al. (1994) studied steroid hormone excretion in the leopard cat, *Felis bengalensis,* cheetah, *Acinonyx jubatus,* clouded leopard, *Neofelis nebulosa,* and snow leopard, *Panthera uncia,* using the domestic cat as a model species. The domestic cat was injected with radio-labelled androgens, with >90% of radioactivity recovered in faeces (Brown et al., 1996).

Several authors have also tested for a correlation between serum and faecal hormone concentrations, to validate the use of faecal steroids as a tool in studying an animal's endocrinology. Although there is usually a time delay between serum hormones and faecal hormones, several studies have shown a good correlation between the two, for example with wombats, *Vombatis ursinus* and *Lasiorhinus latrifrons* (Paris et al., 2002), and macaques, *Macaca fuscata* (Fujita et al., 2001).

There are currently no Ethiopian wolves held in captivity (Sillero-Zubiri & Macdonald, 1997), and handling and restraining wild Ethiopian wolves is kept to a minimum due to their rarity. For this reason, assessing the route of hormone excretion directly or comparing serum and faecal hormone concentrations is not an option in this species. However, several studies have been done in other, closely related species. Testosterone has been found to be excreted in faeces by maned wolves by use of radio-label studies (Velloso et al., 1998), oestradiol and progesterone are excreted in faeces of African wild dogs (tested with radio-label study Montfort et al., 1997), and blood and faecal hormones (oestradiol, testosterone, and progesterone) show correlations in the domestic dog (Gudermuth et al., 1998) Although in domestic dogs only a fraction of cortisol (~23%) is excreted in faeces (Schatz

& Palme, 2001), faecal cortisol metabolite concentrations were found to reflect adrenocortical activity quite well and Schatz and Palme (2001) found that measuring faecal cortisol metabolites was a feasible non-invasive way of studying cortisol excretion in dogs. Faecal cortisol has also been used successfully to study stress in wild grey wolves (Sands & Creel, 2004).

Materials and Methods

1.7 Study Area

The Bale Mountains National Park (BMNP), situated in Southern Ethiopia (7°N, 39°40' E) was established in 1971 (Fig. 1.1). The park covers approximately 2,200km^2, and includes areas ranging in elevation from 1,500 to 4,377m a.s.l. BMNP includes several habitat types including lowland forest and Afroalpine (Hillman, 1988). With over 1,000 km^2 of Afroalpine habitat represented, the Bale Mountains constitute the largest Afroalpine plateau in Africa (Sillero-Zubiri et al., 1998). At the higher altitudes of the park, the climate is characterized by an eight month wet season, with rainfall maxima of 1,150mm per year, and a four month dry season. Temperatures in the dry season range from -15°C at night to +26°C during the day, although temperature fluctuations are more modest during the wet season (Hillman, 1988). The Bale Mountains are home to a large number of endemic species, including not only Ethiopian wolves but also other mammals such as the mountain nyala, Starck's hare, and the giant molerat (Yalden & Largen, 1992). Endemic birds in Bale include spot-breasted lapwing, *Vanellus melanocephalus*, and blue-winged goose, and Bale is also an important breeding area for the red listed wattled crane, *Bugeranus caruncalutus* (Burke, 1996). Endemic plants include the giant lobelia, and fourteen amphibian species are known from the area, with as many as eleven endemics (Hillman, 1988).

This study was carried out in two Afroalpine areas in the BMNP, the Web Valley and the Sanetti Plateau. Both areas have high wolf densities and both include core packs that have been monitored by the Ethiopian Wolf Conservation Programme (EWCP) since 1988. The Web Valley is located at 3,450 to 3,550m a.s.l (Marino, 2003a). The vegetation is typical of Afro-alpine meadowland and is dominated by short alpine grasses and herbs, including African sage, *Artemisia afra*, *Alchemilla spp.*, and bushy everlasting, *Helichrysum splendidum*. Areas with poor drainage are swampy and dominated by tall grasses such as *Cyperus* and *Scirpus* (Malcolm, 1997). The landscape is also dotted with endemic giant lobelias and *Erica* bushes. The Web Valley supports a wolf density of up to 1.2 adult wolves/km^2 (Sillero-Zubiri et al., 1996a), although rabies outbreaks have periodically reduced this density, for example in 2003 (Randall et al., 2004) and more recently in 2008/2009 (Johnson et al., 2010).

The Sanetti Plateau is located at 3,800 to 4,300m a.s.l and includes the park's highest point, Tullu Deemtu (4,377m a.s.l). The Sanetti Plateau is a relatively flat, open area characterized by Afroalpine lakes (Marino, 2003a; Hillman, 1988). The vegetation includes high densities of *Helichrysium splendidum* and giant lobelias (Tallents, 2007). Wolf densities are comparable to those in the Web Valley (Sillero-Zubiri et al., 1996a), and Sanetti wolf densities have been similarly affected by disease outbreaks (see Marino et al., 2006).

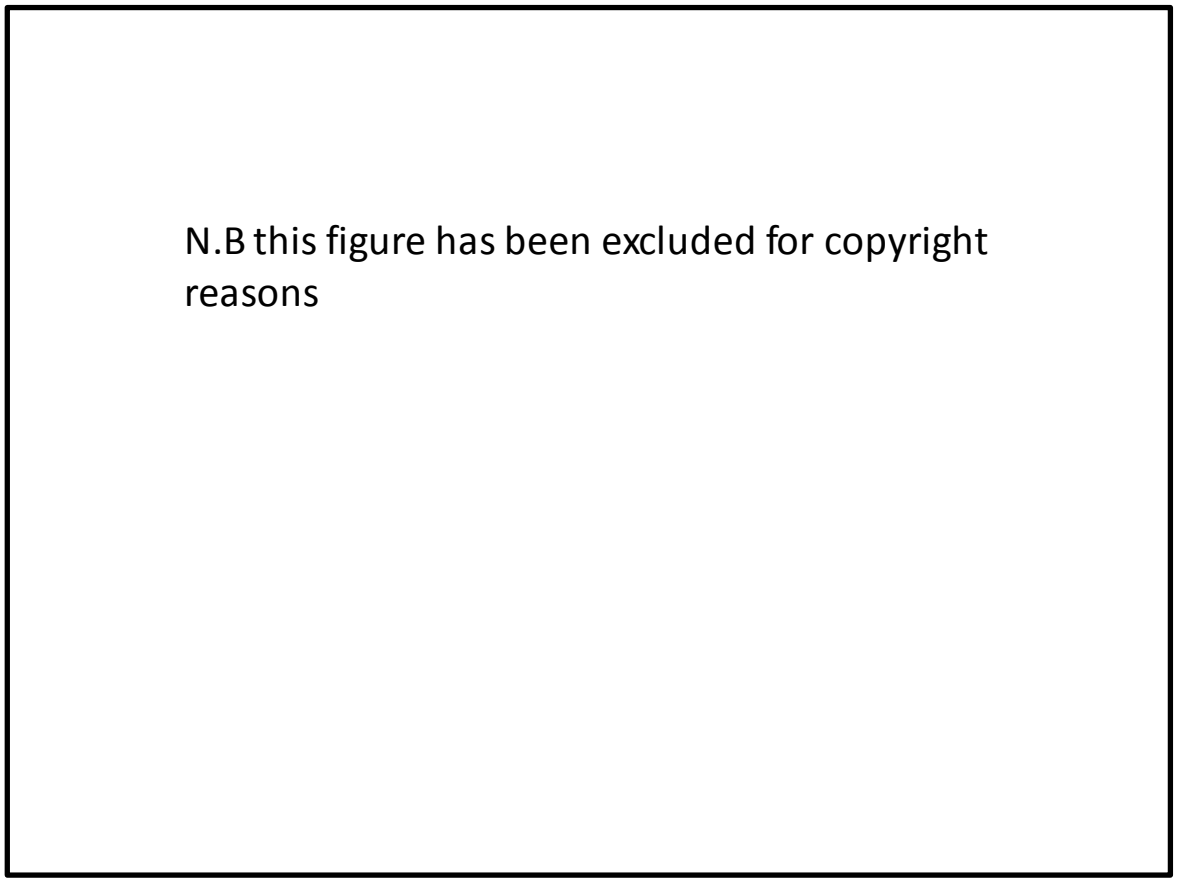

Figure 1.1: Location of the Bale Mountains in Ethiopia (map adapted from Marino, 2003b)

1.8 Study Population

Six packs in the Web Valley and three packs in the Sanetti Plateau were selected for this study (see Fig. 1.2). The Web Valley packs included Addaa, Darkeena, Kotera, Megity, Mulamo and Sodota packs. Although most packs were included for one field season, some packs were included for more than one field season (e.g. Sodota pack, included for three field seasons, Darkeena and Megity packs included for two field seasons). In 2008 and 2009, the Ethiopian wolf population in Web Valley was affected by a rabies epidemic (Johnson et al., 2010), resulting in a ~76% mortality rate in the focal packs (personal observation). Because of this high mortality, focal pack compositions changed substantially (Table 1.1). Pack compositions at both the start and the end of the field season are given for the 2008-2009 field season, to account for the high mortality resulting from the rabies epidemic. Since several females were included for two or three years, the

resulting data will be discussed in pack years. For example, the Sodota dominant female became pregnant and reared pups three times during the course of this study, and this will be referred to as three instances of pregnancy and birth. In Sanetti three packs were selected for this study. These were BBC, Dumal and Nyala packs. Each of these packs was studied for one field season.

To summarize, per field season the following packs were studied:

Field season 1: August 2007-February 2008: Addaa, Darkeena and Sodota packs

Field season 2: August 2008-March 2009: Addaa, Darkeena, Kotera, Megity, Mulamo and Sodota packs

Field season 3: August 2009-February 2010: Megity, Sodota, BBC, Dumal and Nyala packs

Table 1.1: Composition of Ethiopian wolf packs included in this study

	Adults		Juveniles			Pups			
Pack and year	**Female**	**Male**	**Female**	**Male**	**Unknown**	**Female**	**Male**	**Unknown**	**Total**
Web Valley									
Addaa 2007	1	1	0	0	0	0	0	0	2
Addaa August 2008	1	1	0	0	0	0	0	0	2
Addaa March 2009	0	1	0	0	0	0	0	0	1
Darkeena 2007	1	5	3	1	0	0	0	0	10
Darkeena August 2008	4	6	0	0	0	0	0	0	10
Darkeena March 2009	0	1	0	0	0	0	0	0	1
Sodota 2007	1	3	0	0	3	3	0	0	10
Sodota August 2008	1	3	3	0	0	0	0	0	7
Sodota March 2009	1	2	1	0	0	0	0	6	10
Sodota 2009	2	2	3	1	2	0	0	3	13
Kotera August 2008	1	2	0	0	0	0	0	0	3
Kotera March 2009	0	1	0	0	0	0	0	0	1
Megity August 2008	5	12	0	0	6	0	0	0	23
Megity March 2009	2	1	0	0	0	0	1	0	4
Megity 2009	1	3	0	0	0	0	0	5	9
Mulamo August 2008	3	3	0	0	0	0	0	0	6
Mulamo March 2009	1	2	0	0	0	0	0	3	6
Sanetti Plateau									
BBC 2009	3	10	0	0	4	0	0	3	20
Dumal 2009	2	3	1	2	0	0	0	3	11
Nyala 2009	2	3	0	3	1	0	0	0	9

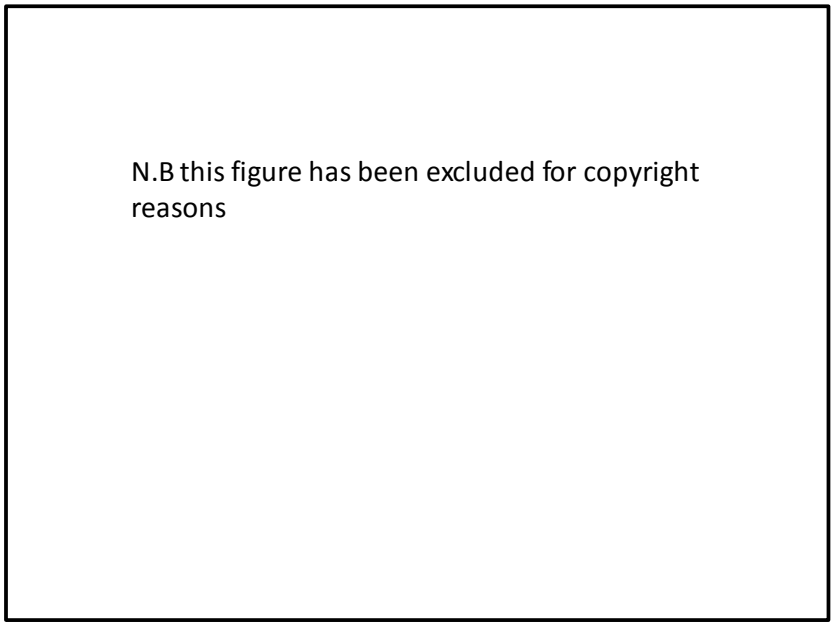

Figure 1.2: Schematic representation of pack locations in Web Valley and Sanetti Plateau. Adapted from Randall (2006)

During the first field season, observation and sampling efforts focused on both adult males and females in the three focal packs Addaa, Darkeena and Sodota. There was one adult male in Addaa pack, five adult males in Darkeena pack and three adult males in Sodota pack. During the second and third field seasons, observation and sampling efforts were concentrated on focal females. For this reason, no males were regularly sampled, but samples from males were collected opportunistically from all packs during the second and third field seasons.

1.9 Field Methods

Packs were selected for this study based on pack compositions (i.e. presence of a breeding female and subordinate females) and for practical reasons (e.g. location of the pack territory in relation to the research camps). Several wolves in the Web Valley and Sanetti Plateau (e.g. SOD02, BBC32, NYA36) had been trapped and ear tagged in EWCP rabies vaccination interventions (e.g. Stewart et al., 2010), and packs with ear tagged individuals were included where possible for identification purposes. Untagged wolves were identified

by individual markings. Dominance hierarchies were often known from long term monitoring by EWCP. Hierarchies in known packs were confirmed and hierarchies in new packs were established by observing interactions between wolves of the same pack. Signs of submission in Ethiopian wolves are similar to those in domestic dogs and grey wolves (Sillero-Zubiri & Gottelli, 1994). Submissive behaviours include tail wagging, laying down and exposing the belly, assuming a crouching position and lowering the ears (Schenkel, 1967). If one identified wolf consistently acted submissive to another in interactions, it was determined to be subordinate to that individual (Sillero-Zubiri & Gottelli, 1994). Using this method, the dominance status of all adult wolves was established.

Packs were followed from August to February or March, in order to include a few weeks of data collection prior to the start of the mating season, as well as a few months after the birth of the pups. Pack territory locations and boundaries were known from concurrent monitoring by EWCP. Since Ethiopian wolves greet and patrol around their territory boundaries early in the morning, and often defecate on these patrols (Sillero-Zubiri & Macdonald, 1998, personal observation), efforts were made to find the wolves early in the morning (6:00-7:00) before they started their patrol. Wolves were found by scanning the landscape with 8x32 binoculars. Once wolves were located they were followed on foot or horseback. Behavioural observations were recorded every 15 minutes using the EWCP protocol. Observations recorded included the time and date of the observation, the number of wolves present, their age and sex categories, the GPS position of the observer, and the distance and compass direction of the wolf from the observer (this was later used to calculate the GPS position of the wolf recorded). The wolves' behaviours were recorded using behavioural categories developed by EWCP (Sillero-Zubiri, 1994). Where relevant,

longer descriptions were added. If any noteworthy behaviour (such as interactions with other wolves) occurred between the 15 minute intervals, these were also recorded. All reproductive behaviours were recorded, including females standing tail aside, males sniffing/licking females' genitals, mounts and copulatory ties. Observations of females' appearance were also recorded, for example if they appeared visibly pregnant or were visibly lactating. Each pack was visited on a regular schedule two or three times a week, in an effort to standardize sampling frequencies. However, sampling frequencies could not be completely standardized in the wild population studied here.

When wolves defecated, the time of defecation was recorded, as well as the time that the sample was collected. Samples were collected on average six minutes after defecation (standard error ±5 minutes) and 96% of samples were collected within 15 minutes of defecation. The age, sex and identity of the wolf were recorded (if known) and a GPS coordinate of the sample was taken. Samples were mixed thoroughly by hand, since concentrations of hormones may vary within a sample (Wasser et al., 1996). Wherever possible only a subsample of the faeces was collected and the rest was left behind, since faeces may serve as olfactory cues (Sillero-Zubiri & Macdonald, 1998). Once mixed, the subsample was stored in a cool box on ice until return to the camp. This was done to prevent bacterial degradation of the hormones inside the sample, and is a method that has been used by several authors to preserve samples in the field until processing (Fujita et al., 2001; Garnier et al., 2002; Ostrowski et al., 2005). Once at the camp samples were processed for preservation. During the first field season (August 2007 – February 2008) 3 grams of wet sample were frozen at -20°C. These samples were later transported to the Veterinary University in Vienna and the MRC Human Reproductive Sciences Unit in Edinburgh on dry ice. During the second and third field seasons (August 2008 – March

2009, August 2009 – February 2010) 3 or 4 grams of wet sample were desiccated in a Coleman® camping oven, at approximately 100°C for one hour, using kerosene heat. Desiccated samples were stored at room temperature and later shipped to Edinburgh at room temperature.

1.10 Extraction of steroids from faeces

We extracted hormones from faecal samples by adding 4ml of analytical grade methanol and 0.5ml of distilled water to each faecal sample (0.5g of wet sample, 0.2g of dry sample), and grinding the sample manually with a pestle and mortar. Ground samples were transferred into glass Pyrex tubes (14 x 100mm) and were shaken in an IKA-Vibrax® XVR orbital shaker for 45 minutes at 1400 r.p.m. After shaking, samples were centrifuged at 2500 r.p.m. at 4°C for 20 minutes. The resulting supernatant was decanted into 10ml glass vials. 4ml of analytical grade methanol and 0.50ml of distilled water were subsequently added to the remaining pellet, which was shaken for a further 45 minutes at 1400 r.p.m and again centrifuged for 20 minutes at 2500 r.p.m. The resulting supernatant was added to the first supernatant and vortexed briefly. 0.50ml of supernatant was pipetted into a 10 x 74 mm glass tube, and this was evaporated under mild heat and nitrogen. Extracts were reconstituted in 1.0ml PGBS assay buffer (7.75g citric acid (Sigma, C7129), 17.85g Na_2HPO_4 (Sigma, S9763) and 1.00g gelatin (Sigma, G9382) in 1L de-ionised water, with 0.10g thiomersol (Sigma T5125), adjusted to pH 6.0) and were stored at 4°C between analyses.

To validate this extraction method, 45 samples were extracted twice (Fig. 1.3), and a subset of 10 samples were extracted a third time (Fig 1.4). The progesterone in each extract was measured using a progesterone RIA protocol (see below). The total progesterone extracted over three extractions was assumed to be 100%. This method showed that a single

extraction yielded on average 70.6% of the progesterone in a sample (standard error 3.9%), and a second extraction yielded a further 21.8% of the progesterone (standard error 3.6%), or a total of 92.4% of the total progesterone in the sample, which was deemed sufficiently efficient (Fig. 1.5).

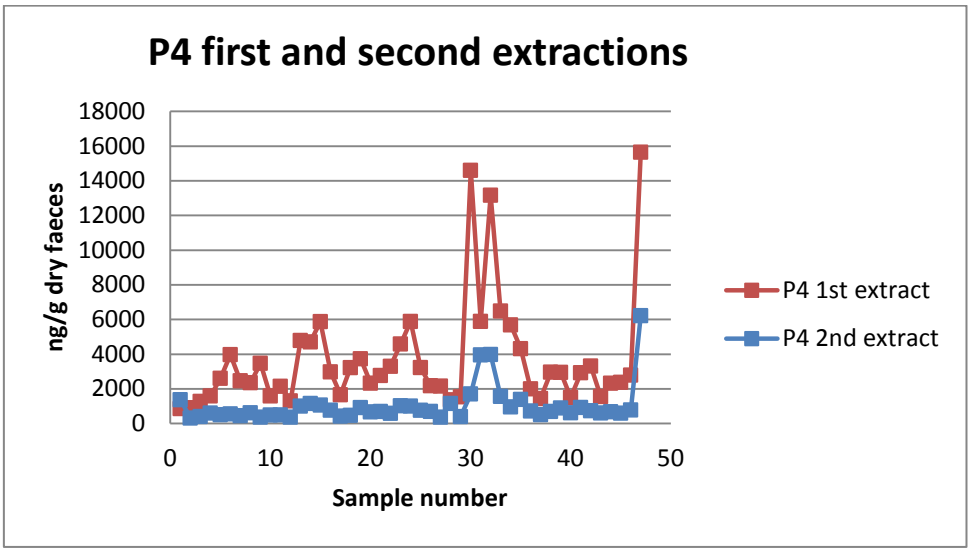

Figure 1.3: Progesterone (P4) yielded by the first and second extractions of 45 samples

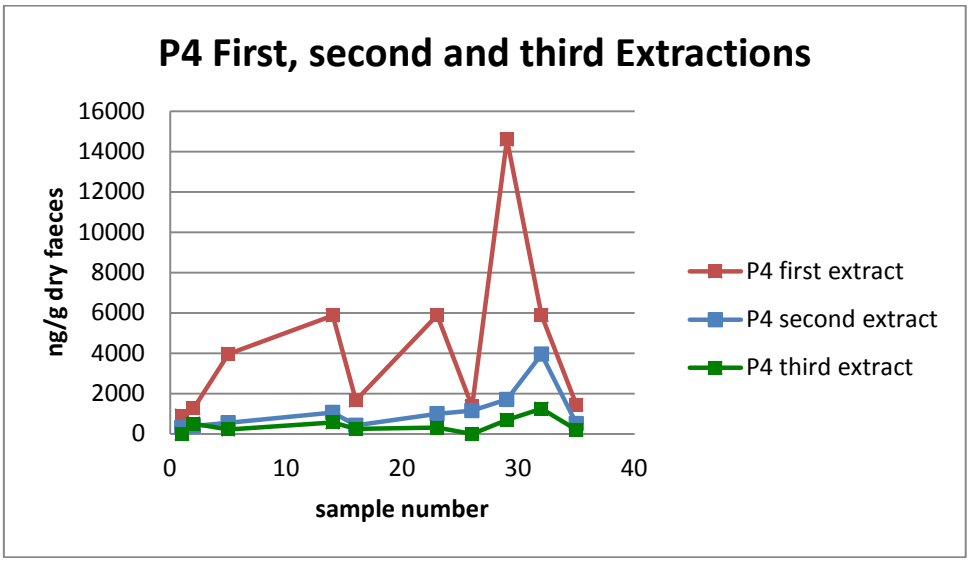

Figure 1.4: Progesterone (P4) yielded by the first, second and third extractions of 10 samples

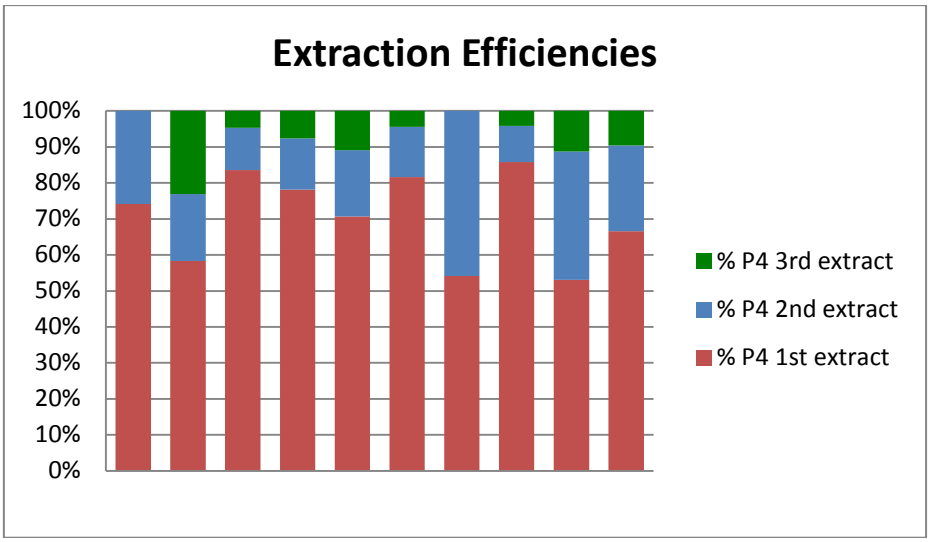

Figure 1.5: Percent of progesterone (P4) obtained by subsequent extractions

1.11 Progesterone, Cortisol and Testosterone Radio-Immunoassay

Background

The progesterone, cortisol and testosterone radio-immunoassays (RIAs) are competitive binding immunoassays. This method of RIA requires labelled antigen, which competes with unlabelled antigen in a sample for a limited amount of antibody, and a method for separating antibody-bound and free labelled antigen (Schuurs & van Weemen, 1980). Samples were added to tubes, and rabbit anti progesterone/cortisol/testosterone antibody was added (primary antibody). Labelled progesterone/cortisol/testosterone (tracer) was added to the tubes, and this competes with the progesterone in the samples. The higher the progesterone/cortisol/testosterone levels in the sample, the lower will be the amount of labelled progesterone/cortisol/testosterone bound to the primary antibody. Following an incubation period, secondary antibody was added to the tubes. This binds to the progesterone/cortisol/testosterone-primary antibody complex. Normal rabbit serum was also added to bulk up the primary antibody-secondary antibody complexes. This creates a larger complex, which precipitates more easily. Following a second incubation, wash

buffer was added to the sample. Wash buffer contains PEG which helps the production of a precipitate. Samples were centrifuged for 30 minutes at 3000 r.p.m. The supernatant was then decanted, leaving only the precipitate pellet inside the tubes. The level of radioactivity in the pellet was then measured using a gamma counter. A high level of radioactivity in the tubes corresponds with a low level of progesterone/cortisol/testosterone in the sample (Nichols, 2009; Becker & Breeddlove, 2002).

Protocol

The specific progesterone, testosterone and cortisol radio-immunoassays used in this study were in-house assays at the MRC Human Reproductive Sciences Unit in Edinburgh. Progesterone, cortisol and testosterone assays were carried out using 75 x 10mm plastic tubes (Sarsted, Germany), with duplicates of every sample. For the progesterone and testosterone assays, 10µL of sample was added to each tube together with 40µL of PGBS assay buffer, and a further 150µL of PGBS assay buffer was added. For cortisol, initial trials using 10µL of sample found that this yielded values that were often below the assay's detection threshold, so instead 100µL of sample was used, with 100µL of PGBS buffer. For progesterone and cortisol, 50µL of standard solutions were used, and 150µL of PGBS assay buffer was added to the standards. For cortisol, 100µL of standard solution and 100µL of PGBS buffer were used. The progesterone standard curve was made up of solutions of 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 ng of progesterone/ml. The testosterone standard curve was made up of solutions of 0.25, 0.5, 1, 2, 4, 8, 16, 31.2, 62.5, 125, 250, 500 ng of testosterone/ml. The cortisol standard curve was made up of solutions of 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500, 1000 and 2000 ng of cortisol/ml. 100µL of primary antibody was added to each sample and standard. 100µL of tracer was added to

each sample and standard. For progesterone the antibody used was raised against 11α-hydroxyProgesterone heminsuccinyl-BSA, Pantex, Santa Monica, USA, and the tracer used was 11-hydroxytyramine-I^{125}, MP-Biomedicals, UK. For cortisol the antibody used was raised against rabbit α cortisol-3, Fitzgerald, USA and the tracer used was ^{125}I labelled-3 MP Biomedicals, UK. For testosterone the antibody used was raised against rabbit anti testosterone-19, AMS Biotechnology, USA and the tracer used was testosterone-19 I^{125}, MP Biomedicals, UK. This information is summarized in Table 1.2. Antibody cross reactivities are given in Tables 1.3, 1.4 and 1.5.

Table 1.2: Progesterone, cortisol and testosterone RIA summary

Assay	Volume of extract assayed	Volume of buffer added to samples	Volume of standards used	Primary antibody	Tracer	Standard curve concentrations
Progesterone	10 µL	190 µL	50 µL	11α-hydroxyProgesterone heminsuccinyl-BSA, Pantex, Santa Monica	11-hydroxytyramine-I^{125}, MP-Biomedicals, UK	3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500, 1000
Cortisol	100 µL	100 µL	50 µL	Rabbit α cortisol-3, Fitzgerald, USA	^{125}I labelled-3 MP Biochemicals, UK	3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500, 1000, 2000
Testosterone	10 µL	190 µL	100 µL	Rabbit anti testosterone-19, AMS Biotechnology, USA	Testosterone-19 I^{125}, MP Biomedicals, UK	0.25, 0.5, 1, 2, 4, 8, 16, 31.2, 62.5, 125, 250, 500

Table 1.3: Progesterone antibody cross reactivities (Pantex, 2010)

Progesterone antibody cross reactivity	**11α-hydroxyProgesterone heminsuccinyl-BSA, Pantex, Santa Monica**
Progesterone	100.00%
17-αHydroxyprogesterone	0.45%
Pregnenolone	0.03%
17-OH-Pregnenolone	<0.001%
Desoxycorticosterone	1.75%
11-Desoxycortisol	0.028%
Corticosterone	0.70%
Aldosterone	0.006%
Cortisol	0.061%
Androstenedione	0.061%
Testosterone	0.017%
5α-Dihydrotestosterone	0.004%
Dehydroepiandrosterone SO4	0.008%
Androstanedione	0.04%
Estradiol-17β	<0.001%
Estradiol-17α	<0.001%
Estriol	<0.001%
Estrone	<0.001%

Table 1.4: Cortisol antibody cross reactivities (Fitzgerald Industries International, 2010b)

Cortisol antibody cross reactivity	**Rabbit α cortisol-3, Fitzgerald, USA**
Cortisol	100.00%
Prednisolone	36.00%
Corticosterone	3.30%
Cortisone	<0.70%

Table 1.5: Testosterone antibody cross reactivities (OEM-Concepts, 2010)

Testosterone antibody cross reactivity	**Rabbit anti testosterone-19, AMS Biotechnology**
Testosterone	100.00%
Dihydro testosterone	1.00%
Nortestosterone	<1.00%
Sex hormone binding globulin	0.50%
Progesterone	0.00%
Other steroids	<0.010%

Assays consisted of:

-Total Counts (TCs) consisting of only 100µL of tracer. TCs give the maximum radioactivity count.

-Non-specific binding (NSBs) consisting of 300µL of PGBS buffer and 100µL of tracer. NSBs do not contain any primary antibody and as such give a measure of background signal.

-B0s consisting of 200 µL PGBS buffer, 100µL tracer, and 100µL of primary antibody. These samples contain no sample or standards and therefore provide a measure of maximum binding in the absence of competition for binding.

-Standard curve

-Quality controls (QCs) consisting of two or three samples which had been previously assayed

-Samples consisting of 10µL or 100µL of sample extract

Once samples, standards, QCs, TCs, NSBs and B0s had been set up and PGBS buffer, tracer and primary antibody had been added where necessary, samples were vortexed briefly and then incubated for at least 3 hours at room temperature or overnight at 4°C.

After the first incubation, 100µL of secondary antibody (donkey anti rabbit serum, DARS, Diagnostics Scotland, 1:60 dilution) and 100µL of Normal Rabbit Serum (NRS, SAPU, UK 1:600 dilution) was added to each tube, except the TCs. All tubes were again vortexed briefly and incubated for at least 3 hours at room temperature of overnight at 4°C. After the second incubation, 1ml of wash buffer (4% w/v PEG, 0.2% v/v Triton-X (Sigma X-100) and 0.9% w/v saline (sodium chloride, Sigma, USA) was added to each tube, except TCs, followed by 45 minutes of centrifugation at 3000 r.p.m. The resulting supernatant was then decanted and tubes were air dried upside down for a few minutes. The radioactivity in each tube was measured using a 1470 Automatic Gamma Counter (Perkin-Elmer, UK). The resulting data was analyzed using Assay-Zap® software (Universal Assay Calculator for Windows, Biosoft, 1996). For each sample, the results are presented as nanograms of hormone per gram of wet or dry faeces.

1.12 Oestradiol Enzyme Immunoassay

Background

The oestradiol assay used was a competitive enzyme immunoassay (EIA). EIA is one of a group of binding assays in which the recognition properties of antibodies are used (Wisdom, 1976). EIAs make use of an antibody labelled in some way, and the labelled component of the antibody binds to its complementary binding site. In competitive EIAs, the antigen in the unknown sample competes with labelled antigen to bind with antibodies. The enzyme activity in either the bound or free fraction is determined and related to the concentration of the unlabeled antigen (Wisdom, 1976). Plates were coated with donkey anti-rabbit antibody, which binds to the surface of the plate, incubated overnight and then washed. Samples and standards were added to wells, and oestradiol antibody was added. Oestradiol in the standards and samples then binds to the oestradiol antibody. Horseradish

peroxidase (HRP) labelled oestradiol was then added to the wells, which competes with the oestradiol in the samples. After a three hour incubation at room temperature, or overnight incubation at 4°C, the plates were washed and substrate for the HRP enzyme was added. This produces a colour reaction, which was stopped by adding 6% phosphoric acid after about 30 minutes. Plates were read with a plate reader which measures the colour intensity of each well. A darker colour corresponds with a low level of oestradiol in the sample.

Protocol

The specific oestradiol enzyme immunoassay used in this study was an in-house assay at the MRC Human Reproductive Sciences Unit in Edinburgh. On the night prior to the assay, 96 well plates were coated with 100µL of Donkey anti-rabbit antibody (in house preparation: Donkey Anti-Rabbit Serum, SAPU, UK mixed with 0.06M acetate buffer, adjusted to pH4.8, centrifuged and filtered through Whatman grade 1 filter paper, used at a 1:60 dilution), diluted 1:100 in coating buffer (1 tablet of calcium bicarbonate buffer, Sigma, USA, in 100ml of distilled water). Plates were then incubated overnight at 4°C. The next day plates were washed three times with plate wash buffer (1 ml Tween®20, Sigma, USA, 999 ml deionised water). On the third wash, the wash buffer was left in the wells for 10 minutes before discarding to remove excess donkey anti-rabbit antibody. The first wells were NSBs containing 150µL of PGBS buffer and 50µL of HRP and B0s containing 100µL of PGBS buffer, 50µL of antibody and 50µL of HRP. The next wells were for the standard curve, with solutions ranging from 0.98 to 125 pg/100 µL. For samples, 10µL of sample with 90µL of PGBS buffer was added to each well. 50µL of antibody was added to each sample and the standard curve (Table 1.6). The antibody used was rabbit anti oestradiol-6, Fitzgerald, USA. Antibody cross reactivities are given in Table 1.7. 50µL of oestradiol-6-HRP conjugate (in house: oestradiol-6-(o-

carboxymethyl)oxime, Sigma K2126 5mg, isobutylchloroformate, Aldrich, 25ml, tri-n-butylane, Aldrich, 50ml, N,N-dimethylformamide, Aldrich, 100ml and horseradish peroxidase, Type VI-A, Sigma, 10mg) was added to each sample and the standard curve. Plates were then incubated for at least 3 hours at room temperature or overnight at 4°C.

Table 1.6: Summary of the oestradiol EIA

Assay	Volume of extract assayed	Volume of buffer added to samples	Volume of standards used	Primary antibody	HRP	Standard curve concentrations
Estradiol	10 µL	90 µL	100 µL	rabbit anti estradiol-6, Fitzgerald, USA	In house estradiol-6-HRP conjugate	0.98, 1.95, 3.9, 7.8, 15.6, 31.2, 125

Table 1.7: Oestradiol antibody cross reactivities (Fitzgerald Industries International, 2010a)

Estradiol antibody cross reactivity	**rabbit anti estradiol-6, Fitzgerald, USA**
Estradiol	100.00%
Estrone	<2.00%
Estriol	<0.15%
Testosterone	<0.01%
DHEA	<0.01%
Andtostenedione	0.00%

Assays consisted of:

-NSBs containing 150µL of PGBS assay buffer and 50µL of Horseradish Peroxidase labelled Oestradiol (HRP-E2). NSBs do not contain any primary antibody and as such give a measure of background signal.

-B0s containing 100µL of PGBS assay buffer, 50µL of oestradiol antibody and 50µL of HRP. These samples contain no sample or standards and therefore provide a measure of maximum binding in the absence of competition for binding.

-Standards consisting of 100µL of oestradiol standard solutions. Standard solutions used were 0.98, 1.95, 3.9, 7.8, 15.6, 31.2, 62.5 and 125 pg/100µL. 50µL of antibody and 50µL of HRP was added to each standard.

-Samples consisting of 10µL of sample in 40µL PGBS assay buffer. 50µL of PGBS assay buffer was added to each sample. 50µL of antibody and 50µL of HRP was added to each sample. All samples were assayed in duplicate.

After incubation, 100µL of TMB was added to each well (mixed equal volumes of TMB peroxidase substrate and peroxidase solution B, TMB Microwell Peroxidase Substrate System, KPL, USA). After about 30 minutes the reaction was stopped by adding 100µL of 6% phosphoric acid to each well. Plates were briefly shaken and read on a MTX Lab Systems Multiscan EX at 450nm. The resulting data was analyzed using Assay-Zap® software (Universal Assay Calculator for Windows, Biosoft, 1996). For each sample, the results are presented as nanograms of hormone per gram of wet or dry faeces.

1.13 Assay validation

Several steps were taken to ensure that the assays were carried out accurately. All samples were assayed in duplicate, and if the two duplicates showed more than 10% variation, the sample was re-assayed. Similarly, outliers were re-assayed to ensure these were not due to laboratory errors. Also, if the standard curve was atypical the analysis was repeated. For progesterone, cortisol and testosterone assays, quality controls were selected from previously assayed samples, and samples of high, medium and low values were chosen (or for cortisol, high and low values). In addition to the quality controls, inter and intra assay controls were carried out for each assay. For inter assay controls, samples that had been previously assayed were randomly selected and inserted into a new assay. The values yielded by the same samples across assays were then compared, and the coefficient of variance was calculated. For intra assay controls, random samples were selected and repeated within the same assay. The values yielded by the same samples were then compared, and the coefficient of variance was calculated. For oestradiol EIAs, instead of adding QC's more inter and intra assay controls were carried out. Inter and intra assay controls are commonly used to validate assays (e.g. Barrett et al., 2002; Beehner &

Whitten, 2004; Brown et al., 1994). All coefficients of variance were below 15% (Table 1.8).

Table 1.8: Coefficients of variance for inter and intra assay controls of each assay

Progesterone	**n**	**Average CV**
Intra-assay validation	5	5.220
Inter-assay validation	6	10.263
QC 1 (high concentration)	7	10.526
QC 2 (medium concentration)	4	9.082
QC 3 (low concentration)	5	14.292
Cortisol	**n**	**Average CV**
Intra-assay validation	3	6.296
Inter-assay validation	5	4.565
QC 1 (high concentration)	2	1.265
QC 2 (low concentration)	3	5.867
Testosterone	**n**	**Average CV**
Intra-assay validation	3	7.736
Inter-assay validation	8	4.706
QC 1 (high concentration)	4	4.334
QC 2 (medium concentration)	4	7.788
QC 3 (low concentration)	4	7.169
Estradiol	**n**	**Average CV**
Inter-assay validation	15	3.311
Intra-assay validation	10	7.802

As a further method of validating the techniques used, a parallelism study was performed. A sample that yielded a high value of each hormone was chosen and serially diluted. These serial dilutions were then assayed, to see if the samples behaved in the same way as the standard curve. This method is also commonly used to validate assays (e.g. Barrett et al., 2002; Fujita et al., 2001; Montfort et al., 1997; Cavigelli, 1999). The values were log transformed, graphed with the log transformation of the standard curve (Figs. 1.6 and 1.7) and the slopes of the serially diluted samples were compared with the slopes of the standard curve using ANCOVA (Fujita et al. 2001). None of the slopes yielded by serially diluting samples differed significantly from the slope of the standard curve (ANCOVA, oestradiol, $F_{1,8}$=0.01, p=0.927; progesterone, $F=_{1,6}$=0.02, p=0.668; cortisol, $F=_{1,4}$=0.00, p=0.968, testosterone, $F_{1,6}$=0.02, p=0.901).

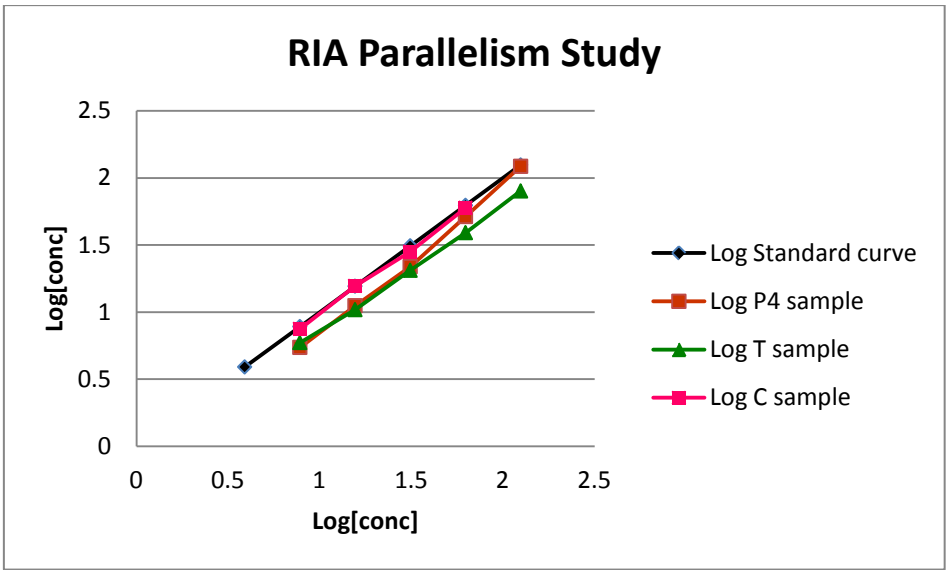

Figure 1.6: Parallelism study for progesterone, testosterone and cortisol

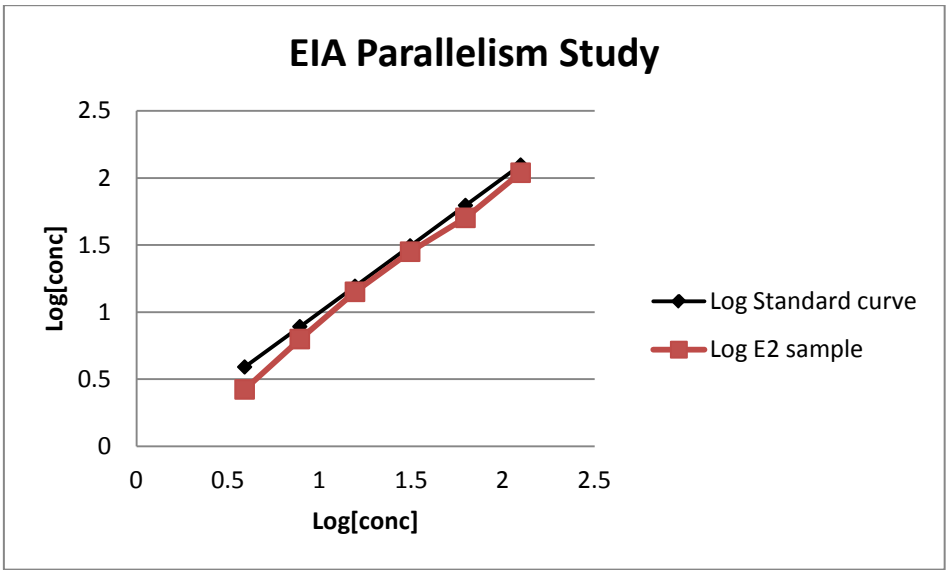

Figure 1.7: Parallelism study for oestradiol

In summary, some of the endocrinological methodology was based on previous studies in closely related species including domestic dogs, (Gudermuth et al., 1998; Schatz & Palme, 2001), as there are no Ethiopian wolves in captivity and the route of hormone excretion in Ethiopian wolves could not be directly established. The sample extraction methodology was validated through a serial extraction experiment, and the immunoassays used were validated through inter and intra-assay controls and parallelism studies. The assay validations indicated that the protocols used were effective for analyzing concentrations of oestradiol, progesterone, testosterone and cortisol in Ethiopian wolf faecal samples.

Chapter 2: Every dog has its way: a review of canid reproductive physiology

Abstract

Advances in reproductive sciences have allowed researchers to study the reproductive physiology of canids in depth. Original research in domesticated species such as domestic dogs, *Canis familiaris*, and Arctic foxes, *Alopex lagopus*, have paved the way for research in other, non-domesticated species including critically endangered red wolves, *Canis rufus*, and endangered African wild dogs, *Lycaon pictus*. This chapter reviews the reproductive physiology studies in all canids studied to date, focusing on the oestrous cycle of females and on seasonal patterns in testosterone and reproductive parameters such as spermatogenesis in males.

2.1 Introduction

The canids form one of the most ubiquitous families of carnivores, with 37 living species that occur throughout most of the world, except Antarctica (Sillero-Zubiri et al., 2004a). The canids include species that are kept and bred by people either for companionship (e.g. domestic dogs) or for their fur (e.g. Arctic fox). Despite this, 20% of all canid species are currently classified as threatened by the IUCN, a further 10% of species are near threatened and a quarter of all wild canid species are declining (IUCN, 2010, see Table 2.1). The Falkland Island fox, *Dusicyon australis*, became extinct in recent history (see Sillero-Zubiri et al., 2004a), and the red wolf, *Canis rufus*, island fox, *Urocyon littoralis*, and Darwin's fox, *Pseudalopex fulvipes*, are currently critically endangered (IUCN, 2010).

Table 2.1: the 37 living canid species. Adapted from Sillero-Zubiri et al. (2004).

Species	Latin name	IUCN status	Population trend	Species	Latin name	IUCN status	Population trend
Arctic fox	*Alopex lagopus*	LC	S	Chilla	*Pseudalopex griseus*	LC	S
Short-eared dog	*Atelocynus microtis*	NT	D	Pampas fox	*Pseudalopex gymnocercus*	LC	I
Side-striped jackal	*Canis adustus*	LC	S	Sechuran fox	*Pseudalopex sechurae*	NT	U
Golden jackal	*Canis aurus*	LC	I	Hoary fox	*Pseudalopex vetulus*	LC	U
Domestic dog	*Canis familiaris*	n/a	n/a	Bush dog	*Speothos venaticus*	NT	U
Dingo	*Canis lupus dingo*	V	D	Gray fox	*Urocyon cinereoargenteus*	LC	S
Grey wolf	*Canis lupus*	LC	S	Island fox	*Urocyon littoralis*	CR	D
Coyote	*Canis latrans*	LC	I	Indian fox	*Vulpes bengalensis*	LC	D
Black-backed jackal	*Canis mesomelas*	LC	S	Blanford's fox	*Vulpes cana*	LC	U
Red wolf	*Canis rufus*	CR	I	Cape fox	*Vulpes chama*	LC	S
Ethiopian wolf	*Canis simensis*	E	D	Corsac	*Vulpes corsac*	LC	U
Crab-eating fox	*Cerdocyon thous*	LC	S	Tibetan fox	*Vulpes ferrilata*	LC	U
Maned wolf	*Chrysocyon brachyrus*	NT	U	Kit fox	*Vulpes macrotis*	LC	D
Dhole	*Cuon alpinus*	E	D	Pale fox	*Vulpes pallida*	LC	U
African wild dog	*Lycaon pictus*	E	D	Rüppell's fox	*Vulpes rueppelli*	LC	U
Raccoon dog	*Nyctereutes procyonoides*	LC	S	Swift fox	*Vulpes velox*	LC	S
Bat-eared fox	*Otocyon megalotis*	LC	U	Red fox	*Vulpes vulpes*	LC	S
Culpeo	*Pseudalopex culpaeus*	LC	S	Fennec fox	*Vulpes zerda*	LC	U
Darwin's fox	*Pseudalopex fulvipes*	CR	D				

LC= Least concern	S= Stable
NT= Near Threatened	D= Decreasing
V = Vulnerable	I= Increasing
E= Endangered	U=Unknown

The domestic dog was the first species to be domesticated by man more than 14,000 years ago (Driscoll et al., 2009). Molecular evidence points to an origin of dogs from grey wolves, but over the last 14,000 years they have evolved into a new species now often referred to as ‘man’s best friend’ (Driscoll et al., 2009). Given this affiliation, it is not surprising that people have long since had an interest in domestic dog reproduction. Indeed the first successful artificial insemination in a domestic bitch was recorded as early as 1770 (Heape, 1897). With the development of hormone assay techniques, detailed reproductive physiology studies became possible. Efforts to understand canid reproductive physiology initially focused on domestic dogs (e.g. Concannon et al., 1977; Concannon et al., 1975), and on animals that are bred in the commercial fur trade, including Arctic (Møller, 1973) and red (Mondain-Monval et al., 1977) foxes. Furthermore, new reproductive technologies such as semen banking, egg cell harvesting and artificial insemination have been developed and used in canids including domestic dogs and captive Arctic and red foxes

(see Farstad, 2000b; Farstad, 2000a; Thomassen & Farstad, 2009). These new research methods, first applied in domesticated species, and the ensuing new knowledge are now benefiting the study of wild species.

It is increasingly recognized that an in-depth understanding of reproduction is necessary for the management of both wild and captive populations (e.g. Graham, 2004), and captive breeding programmes often play an important role in conservation. Captive breeding has recently played an important role in conserving the critically endangered red wolf (see Hedrick & Fredickson, 2008) and island fox (Asa et al., 2007; Coonan et al., 2005). Captive populations of canid species have also been used for reproductive physiology studies, and even more recently wild populations have been studied using non-invasive methods (e.g. Creel et al., 1997a). Thanks to these studies the reproductive physiology of about a third of all canid species has now been studied in detail, including domestic dogs (e.g. Concannon et al., 2009; Concannon et al., 1975; Ortega-Pacheco et al., 2006), grey wolves (Kreeger, 2003; Seal et al., 1979) red wolves, (Walker et al., 2002), coyotes (Kennelly & Johns, 1976; Minter & DeLiberti, 2008; Carlson & Gese, 2008), red foxes (Maurel & Boissin, 1981; Mondain-Monval et al., 1977), Arctic foxes (Sanson et al., 2005; Smith et al., 1985), fennec foxes (Valdespino et al., 2002), island foxes (Asa et al., 2007), maned wolves (Velloso et al., 1998; Wasser et al., 1995), bush dogs (DeMatteo et al., 2006), African wild dogs (Creel et al., 1997a; Johnston et al., 2007; Montfort et al., 1997), and raccoon dogs (Asikainen et al., 2003; Rudert et al., 2010; Valtonen et al., 1977). Furthermore, new reproductive technologies such as semen banking, egg cell harvesting and artificial insemination are now also being applied to wild canids such as red wolves (Goodrowe et al., 1998).

However, despite advances in canid reproduction research, there are still several canid species for which few reproductive data are available, and which are not currently being bred in captivity. These include the near-threatened short-eared dog, the critically endangered Darwin's fox and the Ethiopian wolf, the world's rarest canid (Sillero-Zubiri et al., 2004a), despite recommendations of reproductive management including captive breeding for the latter two species (Sillero-Zubiri & Macdonald, 1997; Yahnke et al., 1996). Fortunately, research in other canids has assisted us in understanding canid reproduction, and can help to formulate studies on new, as yet unstudied species. This chapter reviews the existing literature on reproductive physiology in the canid species studied to date, focusing especially on knowledge gained on reproductive cycles of females and seasonality of reproductive hormones and parameters in males.

2.3 Female Canid Reproductive Physiology

2.3A The oestrous cycle of the domestic bitch

The first studies on female canid reproductive physiology were on domestic bitches, and after years of research the reproductive physiology of the domestic bitch is now well understood. Domestic bitches are generally monoestrous aseasonal breeders (Bouchard, 1991), although they may have two litters in one year (Asa & Valdespino, 1998). A typical interoestrous interval in domestic bitches ranges from 5 to 10 months, with an average of 7.7 months, although oestrous cycle lengths are very variable, even within one bitch (Bouchard, 1991). The reproductive cycle of domestic bitches is generally categorized into four different stages. These are called pro-oestrus, oestrus, metoestrus and anoestrus, and each stage is characterized by different behavioural, anatomic and physiological characteristics (Jöchle & Andersen, 1976).

1. **Pro-oestrus:** Pro-oestrus is the beginning of the sexual season, and lasts 5-15 days in the domestic bitch. Pro-oestrus is characterized by an enlargement of the vulva and sanguineous discharge (Jöchle & Andersen, 1976). Females also become more attractive to males during pro-oestrus (Beach et al., 1982). During pro-oestrus, follicles grow and develop in preparation for ovulation and oestradiol levels rise (Concannon et al., 2009). The increase in oestradiol triggers the LH (luteinizing hormone) release which precedes ovulation during oestrus (Wildt et al., 1979).

2. **Oestrus:** Pro-oestrus is followed by oestrus. The start of oestrus can be established by female acceptance of male mating attempts, or assumption of the breeding stance i.e. standing in front of the male with the tail aside (Jöchle & Andersen, 1976). This oestrus behaviour usually occurs either at or just after the peak in oestradiol (Concannon et al., 2009). Oestrus lasts for 5-15 days in domestic bitches and is characterized by an enlarged vulva, male acceptance and reduced vaginal discharge. During oestrus oestrogens decrease, LH peaks and declines and progestins rise (Jöchle & Andersen, 1976). LH stimulates the last stages of follicular development (Kooistra et al., 1999), and ovulation generally occurs two days after the LH surge (Concannon et al., 1977; Phemister et al., 1973). After ovulation, the corpus luteum (CL) is formed, which produces progesterone (Concannon et al., 2009). The hormonal changes occurring during pro-oestrus and oestrus are shown in Fig 2.1.

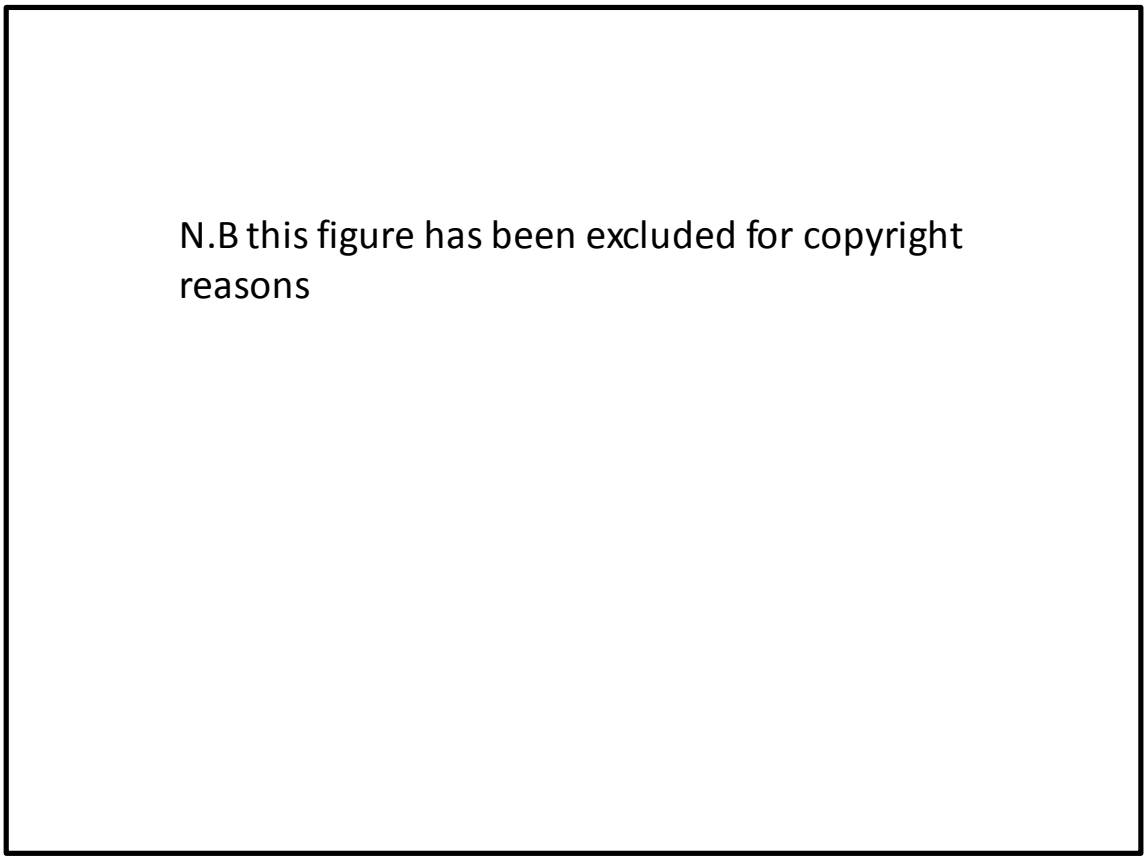

Figure 2.1: Schematic representation of hormone fluctuations and changes in behaviour in a domestic bitch in pro-oestrus and oestrus. Adapted from Feldman and Nelson (1987)

3. **Metoestrus:** Oestrus is followed by metoestrus (also known as dioestrus), which lasts for 130-140 days and can involve pregnancy, whelping and lactation or pseudopregnancy.

   Pregnancy: Pregnancy lasts on average 64 days in Labradors (Chakraborty, 1987) and 61-65 days in Beagles (Concannon et al., 1975). During metoestrus, progestins plateau and decrease. In pregnant bitches, progesterone levels decline sharply shortly before term (Jöchle & Andersen, 1976; Concannon et al., 1978) and studies have shown that parturition can be induced in domestic bitches by blocking the activity of progesterone with an antigestagen (Hoffmann, 1996). In domestic bitches, the CL is crucial for the maintenance of pregnancy as no progesterone is produced by the placenta (Concannon et al., 2009), and indeed, ovariectomy leads to abortion in pregnant bitches (Tsutsui, 1983).

Pseudopregnancy: Domestic bitches often show pseudopregnancy following a non-conceptive oestrus, and pseudo-pregnancy is characterized by hormonal changes including increased progesterone (Chakraborty, 1987; Concannon et al., 2009; Gobello et al., 2001). The CL is crucial for pseudopregnancy, as it is the main source of progesterone in domestic bitches (Concannon et al., 2009) Pseudopregnancy can be overt or covert. Overt pseudopregnancy can be characterized by physiological and behavioural changes such as abdominal distension, enlargement of mammary glands and possible secretion of milk, as well as maternal behaviours (Chakraborty, 1987; Concannon et al., 2009). In contrast, covert pseudopregnancy can occur, without any external manifestations of pseudopregnancy (Smith & McDonald, 1974). Hormonal patterns of both progesterone and LH do not differ between overt and covert pseudopregnancies (Smith & McDonald, 1974). Serum progesterone levels of pregnant and non-pregnant bitches are difficult to distinguish (Concannon et al., 1975), although this is probably due to hemodilution and increased progesterone metabolism (Concannon et al., 2009). Gudermuth et al. (1998) did find higher progesterone levels in the faeces of pregnant bitches as compared to non pregnant bitches.

4. **Anoestrus:** Metoestrus is followed by anoestrus, which is characterized by a slight rise of oestrogens and improved physical appearance, and is a period of reproductive quiescence (Jöchle & Andersen, 1976). Towards the end of anoestrus, follicle stimulating hormone (FSH) concentrations begin to rise. FSH plays an important role in the early stages of follicular development in domestic bitches (Kooistra et al., 1999), and therefore in the termination of anoestrus (Kooistra &

Okkens, 2001). The different stages of the domestic bitch reproductive cycle are shown in Fig 2.2.

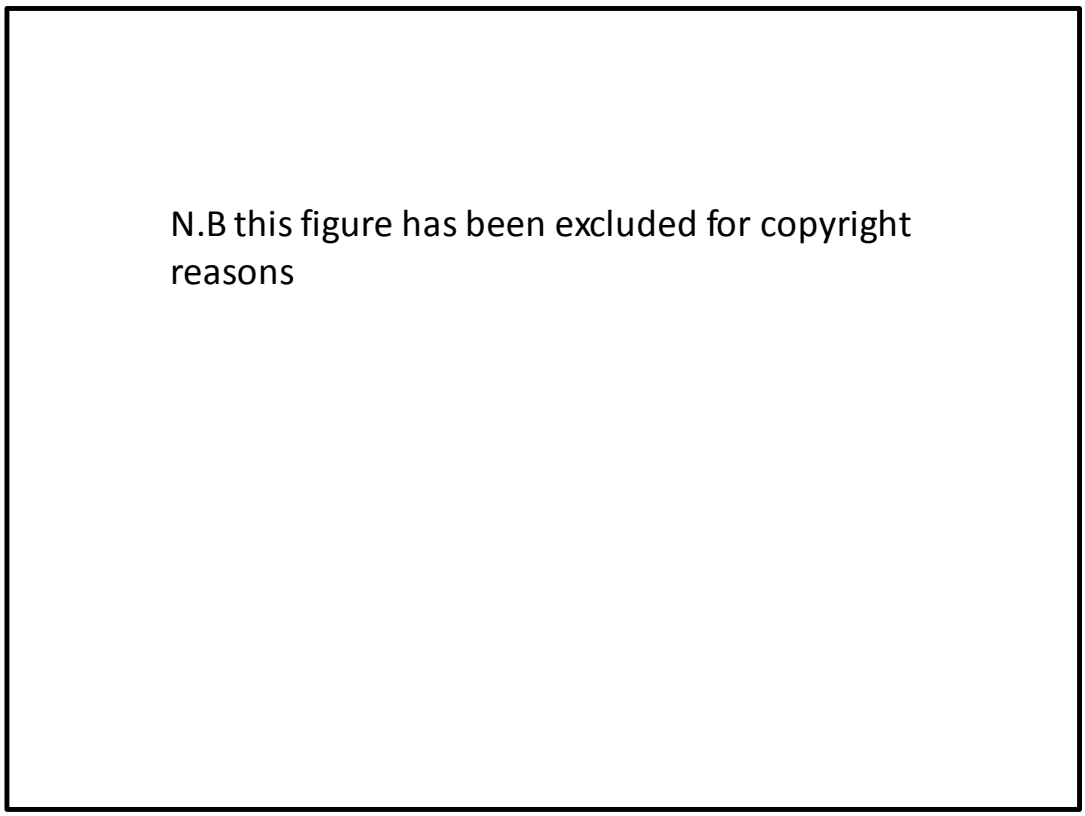

Figure 2.2: The phases of the domestic bitch oestrous cycle. Black (solid) lines represent a non-conceptive cycle and red (dashed) lines represent a conceptive cycle. Adapted from Concannon et al. (2009).

The oestrous cycle of several canids has been studied in detail. These include grey and red wolves, coyotes, red, Arctic, fennec and island foxes, maned wolves, bush dogs, African wild dogs and raccoon dogs (Table 2.2). Most canids breed seasonally, and the reproductive stages described for domestic dogs have been described in all other canid species studied, although the length of the different reproductive stages differ per species.

Table 2.2: Summary of reproductive physiology in females of 12 canid species

Species	Latin name	Seasonal or aseasonal	Length of oestrus	Length of pregnancy	May become pseudo-pregnant?	Reference
Domestic dog	*Canis familiaris*	Aseasonal	5-15 days	59-68 days	yes	Concannon 1975, Jochle and Anderson 1976, Chakraborty 1987
Grey wolf	*Canis lupus*	Seasonal	9 days	63 days	yes	Seal et al 1979, Kreeger 2003
Red wolf	*Canis rufus*	Seasonal	8 days	64-65 days	yes	Walker et al 2002
Coyote	*Canis latrans*	Seasonal	10.3 days	60-63 days	yes	Carlson and Gese 2007, 2008
Maned wolf	*Chrysocyon brachyrus*	Seasonal	4.3 days	65 days	yes	Velloso et al 1998
Bush dog	*Speothos venaticus*	Aseasonal	1-12 days	67 days	yes	Porton et al 1987, DeMatteo et al 2006
Red fox	*Vulpes vulpes*	Seasonal	2-4 days	51-52 days	yes	Mondain-Monval et al 1977, Farstad 1998
Arctic fox	*Alopex lagopus*	Seasonal	4-5 days	52-53 days	yes	Farstad 1998, Sanson et al 2005
Fennec fox	*Vulpes zerda*	Seasonal	1 day	49-51 days	yes	Valdespino et al 2002
Island fox	*Urocyon littoralis*	Seasonal	n/a	50-53 days	unk (induced ovulators)	Asa et al 2007, Roemer et al 2004
African wild dog	*Lycaon pictus*	Seasonal	6-9 days	69 days	yes	Montfort et al 1997, 1998
Raccoon dog	*Nyctereutes procyonoides*	Seasonal	3.9 days	59-64 days	yes	Valtonen et al 1997, Rudert et al 2010

2.3B Grey wolves, red wolves and coyotes

The oestrous cycle of grey wolves (Seal et al., 1979), red wolves (Walker et al., 2002) and coyotes (Carlson & Gese, 2008; Kennelly & Johns, 1976) is similar to that of domestic bitches, including the same reproductive stages. However, grey wolves, red wolves and coyotes are seasonal breeders.

Grey wolves are seasonal monoestrous breeders, and the mating season in North America is between late January and early April (Kreeger, 2003). The length of pro-oestrus in grey wolves was found to be on average 15.7 days, with oestrus lasting 9 days on average and pregnancy lasting 63 days on average. Serum oestradiol levels were similar to those reported for domestic bitches, with oestradiol peaking in late pro-oestrus. As in domestic

bitches, pro-oestrus was characterized by vaginal discharge (Seal et al., 1979). Both pregnant and non-pregnant females showed increases in serum progesterone levels during and after the LH surge, and progesterone levels declined at parturition (Seal et al., 1979).

Red wolves breed once a year, and the mating season is between mid December and late May (Walker et al., 2002). Female red wolves also show similar reproductive patterns as domestic bitches. Oestrus behaviour is characterized by tail deflection and mounting, and is associated with falling oestrogen and rising progestagen concentrations. Gestation length is 64-65 days and both pregnant and ovulatory non-pregnant red wolves showed an increase in progestagen levels following ovulation (Walker et al., 2002), indicating they may become pseudopregnant.

Coyotes differ somewhat from grey and red wolves in that their reproductive cycle is characterized by a long (2-3 month) pro-oestrus. As in domestic dogs, pro-oestrus in coyotes is characterized by vulval swelling and sanguineous discharge (Kennelly & Johns, 1976). Oestrus has been estimated to last on average between 7.6 (Carlson & Gese, 2008) and 10.3 (Kennelly & Johns, 1976) days in coyotes, and ovulation is estimated to take place between days 1-9 of oestrus (Kennelly & Johns, 1976). Pregnancy lasts approximately 60-63 days (see Carlson & Gese, 2007), and serum progesterone levels of pregnant and pseudopregnant female coyotes are indistinguishable (Carlson & Gese, 2008).

2.3C Maned wolves and bush dogs

Reproductive studies in South American canids include maned wolves (Velloso et al., 1998; Wasser et al., 1995), and bush dogs (DeMatteo et al., 2006). Again, the reproductive phases of maned wolves are similar to those of domestic bitches, although maned wolves

are seasonal breeders. Pro-oestrus in maned wolves is characterized by vaginal swelling and discharge and solicitous tail flagging, and lasts an average of 10.6 days. Oestrus (lasting on average 4.3 days) is characterized by an increase in reproductive behaviours, including mating and copulatory ties observed at or shortly after a faecal oestradiol surge. Pregnancy lasts about 65 days and is characterized by an increase in faecal progestins (Velloso et al., 1998). Wasser et al. (1995) studied oestrogen and progestin concentrations in the faeces of nine female maned wolves. Pre-ovulatory peaks of oestrogen were detected in females sampled at the expected time of ovulation, and the four females in the study who became pregnant mated just after this oestrogen peak. Although both pregnant and pseudopregnant female maned wolves showed an increase in faecal progestins, levels of progestins were significantly higher in pregnant females (Velloso et al., 1998; Songsasen et al., 2006).

The bush dog, a small canid from Central and South America, is similar to the domestic dog in that it breeds aseasonally (Porton et al., 1987). Pro-oestrus is estimated to last about 14 days (Porton, 1983). Oestrus in captive bush dogs was found to last between one and twelve days, with pregnancy lasting on average 67 days (Porton et al., 1987). Bush dogs have an obligate pseudopregnancy following a non-conceptive oestrus, during which progesterone levels are increased (DeMatteo et al., 2006).

2.3D Red, Arctic, fennec and island foxes

Sillero-Zubiri et al. (2004a) list 22 living fox species in six genera, including *Vulpes*, the true foxes (see Table 2.1). Of these species, the reproductive physiology of only a few has been studied. These include red and Arctic foxes, which are commonly bred commercially for their fur (Sanson et al., 2005), fennec foxes which are also kept as pets (Asa et al., 2004), and the critically endangered island fox (Asa et al., 2007).

Red foxes are seasonal and monoestrous breeders, and in the wild the breeding season is between January and March (Maurel & Boissin, 1981). Pro-oestrus lasts an estimated 15 days, sexual receptivity in the red fox lasts 2-4 days and pregnancy lasts 51-52 days (Farstad, 1998; Mondain-Monval et al., 1977). Pro-oestrus is characterized by vulval swelling but is not associated with sanguineous discharge (Boue et al., 2000) and ovulation is preceded by an LH surge (Mondain-Monval et al., 1984). Pregnancy in red foxes is associated with increased progesterone (Bonnin et al., 1978) and unmated females showed increased levels of progesterone for between 60-85 days after ovulation, indicating they may become pseudopregnant (Mondain-Monval et al., 1977).

Arctic foxes (also known as blue foxes), like red foxes, are seasonal monoestrous breeders, with a breeding season between March and May in the Northern hemisphere (Farstad, 1998). Pro-oestrus is estimated to last between 12 and 24 days (see Korhonen & Alasuutari, 1992), and is characterized by vulval swelling, but not sanguineous discharge (Farstad et al., 1993; Møller et al., 1984). LH peaks during pro-oestrus (Mondain-Monval et al., 1984). Oestrus in Arctic foxes lasts 4-5 days. Pregnancy lasts 52-53 days, and, in the absence of pregnancy, Arctic foxes may become pseudopregnant (Farstad, 1998; Møller, 1973), although faecal progesterone levels are significantly higher in pregnant than non-pregnant females (Sanson et al., 2005).

Fennec foxes are monoestrous breeders, with mean cyclic intervals of 9.9 months (Valdespino et al., 2002). Like other canids, fennec foxes show an oestradiol increase in pro-oestrus (lasting approximately 6.5 days), and pro-oestrus is also characterized by vaginal swelling, and fennec foxes do not have sanguineous vulval discharge (Valdespino

et al., 2002). Oestrus was found to be extremely short in this species, with mating observed on only one day for nine of ten observed oestrous cycles. Pregnancy lasted 49-51 days. In this study, all mated females became pregnant. Two unmated females showed increased progesterone levels following pro-oestrus, although levels were attenuated compared to pregnant females. Although data is scarce, these observations suggest that fennec foxes may also become pseudopregnant following non-conceptive oestrus (Valdespino et al., 2002).

Island foxes are the smallest North American canid and occur only on the California Channel Islands (Roemer et al., 2004). Island foxes breed once a year with parturition usually occurring in early April. Pregnancy is estimated to last 50-53 days (Roemer et al., 2004). Recent research suggests that island foxes have an induced ovulation instead of a spontaneous ovulation, and that the presence of a male may be required for female island foxes to ovulate (Asa et al., 2007). Induced ovulators, unlike spontaneous ovulators, go through a follicular phase, but then remain in a state of sexual receptivity where the follicles mature and are usually released after copulation (Hadley 2000). This makes island foxes unusual among canids, as other species studied were all spontaneous ovulators (Asa et al., 2007).

2.3E African wild dogs

African wild dogs breed seasonally and are monoestrous (Montfort et al., 1998; Woodroffe et al., 2004), although in some captive populations a second breeding season has been recorded in packs which failed to breed during the main breeding season (Boutelle & Bertschinger, 2010), suggesting that African wild dogs may show some flexibility in their reproduction. In the Southern hemisphere pups are usually born between May and June (McNutt, 1996), although this can shift to December in captive populations held in the

Northern hemisphere (Montfort et al., 1997). Pro-oestrus in African wild dogs is characterized by increased oestradiol levels, and vaginal swelling and discharge (Montfort et al., 1997). Oestrus in wild dogs lasts 6-9 days and oestrus behaviour, including mating, is associated with peak or declining oestrogen and increasing progesterone (Montfort et al., 1997). Oestrus may be followed by a 69 day pregnancy and female African wild dogs who ovulate but do not conceive pups become pseudopregnant, and show increased levels of progesterone (Montfort et al., 1997).

2.3F Raccoon dogs

The raccoon dog is the only living member of the genus *Nyctereutes* (Rudert et al., 2010). Although raccoon dogs are an Asian species, they now occur in many parts of Russia and Western Europe, where they were introduced for their fur (Kauhala & Saeki, 2004b), and they are also bred in fur farms (Asikainen et al., 2003). Raccoon dogs are seasonal breeders and are monoestrous (Asikainen et al., 2003). The mating season in Europe is in March (Rudert et al., 2010) and between February and April in Japan (Kauhala & Saeki, 2004a). Pro-oestrus in raccoon dogs is characterized by vulval swelling and discharge, and lasts on average 7.6 days. During pro-oestrus and early oestrus, oestradiol levels peak (Valtonen et al., 1978). Oestrus lasts an estimated 3.9 days, and can be determined by the female's willingness to mate (Valtonen et al., 1977). Pregnancy lasts between 59 to 64 days (Valtonen et al., 1977) and females may become pseudopregnant and show increased levels of progesterone (Rudert et al., 2010; Valtonen et al., 1978).

Table 2.3: Hormonal, physiological and behavioural characteristics of the reproductive cycle in 11 canid species

	Pro-oestrous					Oestrous				Metoestrous		
	Hormonal changes		**Physiological changes**		**Behavioural changes**	**Hormonal changes**		**Physiological changes**	**Behavioural changes**	**Hormonal changes**	**Physiological changes**	
	E levels rise	LH surge	Vulval swelling	Sanguin-eous discharge	Female attractive to males	LH peaks & declines	P rise	Ovulation	Sexually receptive&mating	P rise, decrease before birth	Pregnancy & birth	Possible pseudo-pregnancy
Domestic dog	✓[1]	✓[1]	✓[1]	✓[1]	✓[2]	✓[1]	✓[1]	✓[3,4]	✓[1]	✓[1,5]	✓[1,5]	✓[6,7]
Grey wolf	✓[8]	✓[8]	*	✓[8]	✓[8]	✓[8]	✓[8]	✓[8]	✓[8]	✓[8]	✓[8]	✓[8]
Red wolf	✓[9]	✓[9]	*	*	*	✓[9]	✓[9]	✓[9]	✓[9]	✓[9]	✓[9]	✓[9]
Coyote	✓[10]	*	✓[11]	✓[11]	✓[10]	*	✓[10]	✓[10,11]	✓[10]	✓[10]	✓[10,11]	✓[10]
Maned wolf	✓[12]	*	✓[12]	✓[12]	✓[12]	*	✓[12]	✓[12]	✓[12]	✓[12,13]	✓[12,13]	✓[12]
Bush dog	✓[14]	*	✓[14]	X[14]	*	*	✓[14]	✓[14]	✓[14]	✓[14]	✓[14]	✓[14]
Red fox	✓[15]	✓[16]	✓[17]	X[17]	*	*	✓[15]	✓[15]	*	✓[18]	✓[18]	✓[18]
Arctic fox	✓[19]	✓[19]	✓[19]	X[20]	*	✓[19]	✓[19]	✓[19]	✓[20]	✓[21]	✓[21]	✓[21]
Fennec fox	✓[22]	*	✓[22]	X[22]	✓[22]	*	✓[22]	✓[22]	✓[22]	✓[22]	✓[22]	✓[22]
African wild dog	✓[23]	*	✓[23]	✓[23]	✓[23]	*	✓[23]	✓[23]	✓[23]	✓[23]	✓[23]	✓[23]
Raccoon dog	✓[24]	*[24]	✓[25]	✓[25]	*	*	✓[24]	✓[26]	✓[25]	✓[24]	✓[24]	✓[26]

✓ Suppored by data
X Not supported by data
* Data not available to the best of the author's knowledge

1 Jochle and Anderson 1976
2 Beach et al 1982
3 Phemister et al 1973
4 Concannon et al 1977
5 Concannon et al 1978
6 Concannon et al 1975
7 Chakraborty 1987
8 Seal et al 1979
9 Walker et al 2002
10 Carslon and Gese 2008
11 Kennelly and Johns 1976
12 Velloso et al 1998
13 Wasser et al 1995
14 DeMatteo et al 2006
15 Mondain-Monval et al 1977
16 Mondain-Monval et al 1984
17 Boue et al 2000
18 Bonnin et al 1978
19 Møller et al 1984
20 Farstad et al 1993
21 Møller1973
22 Valdespino et al 2002
23 Montfort et al 1997
24 Asikainen et al 2003
25 Valtonen et al 1977
26 Rudert et al 2010

Note: *E=oestrogens, LH=luteinizing hormone, P=progestins*

2.4 Male canid reproductive physiology

The main hormone associated with male reproductive physiology is testosterone. Testosterone plays several roles in male development, reproduction and behaviour. For example, in humans, foetal testosterone (together with Müllerian regression hormone) is responsible for the formation of the male phenotype (Wilson et al., 1981), and in adults testosterone is required for normal initiation and maintenance of spermatogenesis (van der Molen et al., 1979). Testosterone has also been found to be required for both simple and complex sexual behaviour. In rats, for example, penile reflexes show great sensitivity to blood concentration of testosterone (Hart, 1983) and in birds, courtship behaviour is largely influenced by testosterone (Fusani, 2008). Studies in birds have shown that testosterone implants can cause normally monogamous males to become polygynous (Wingfield, 1984), and testosterone implants can also shift behaviours from paternal behaviour to mating behaviour (De Ridder et al., 2000).

The role of testosterone in male canid reproduction has been studied in domestic dogs (Ortega-Pacheco et al., 2006), grey wolves (see Kreeger, 2003), red wolves (Walker et al., 2002), coyotes (Minter & DeLiberti, 2008), maned wolves (Velloso et al., 1998), red foxes (Maurel & Boissin, 1981; Forsberg & Madej, 1990), Arctic foxes (Smith et al., 1985), African wild dogs (Johnston et al., 2007) and raccoon dogs (Rudert et al., 2010; Xiao et al., 1995). Seasonally breeding canids show seasonal trends in testosterone levels and/or other reproductive parameters such as spermatogenesis (Table 2.4)

Table 2.4: Summary of reproductive physiology in males of nine canid species

Species	Latin name	Seasonal or aseasonal	Seasonal patterns in testosterone	Seasonal patterns in spermatogenesis	Reference
Domestic dog	*Canis familiaris*	Aseasonal	no	no	Ortega-Pacheco et al 2006
Grey wolf	*Canis lupus*	Seasonal	yes	yes	Kreeger 2003
Red wolf	*Canis rufus*	Seasonal	yes	yes	Walker 2002
Coyote	*Canis latrans*	Seasonal	yes	yes	Minter and DeLiberti 2008
Maned wolf	*Chrysocyon brachyrus*	Seasonal	no	yes	Velloso et al 1998
Bush dog	*Speothos venaticus*	Aseasonal	no	no	Porton et al 1987
Red fox	*Vulpes vulpes*	Seasonal	yes	yes	Maurel and Boissin 1981, Farstad 1998
Arctic fox	*Alopex lagopus*	Seasonal	yes	yes	Smith et al 1985, Farstad 1998
African wild dog	*Lycaon pictus*	Seasonal	no	yes	Creel et al 1997, Johnston et al 2007
Raccoon dog	*Nyctereutes procyonoides*	Seasonal	yes	yes	Xiao et al 1995, Rudert et al 2010

2.4A Domestic dogs

Domestic dogs are unusual among the Canidae because they are aseasonal breeders, capable of breeding throughout the year (Bouchard, 1991). As may be expected from an aseasonally breeding species, male Beagles do not show seasonal patterns in testosterone (Thun et al., 1990). Similarly, stray male domestic dogs in the tropics have been found to have constant serum testosterone levels during the year and produce sperm throughout the year (Ortega-Pacheco et al., 2006).

2.4B Grey wolves, red wolves and coyotes

Grey wolves are seasonal breeders, with one breeding season per year (Seal et al., 1979). Testosterone shows a seasonal pattern in grey wolves, with highest levels between December and March (Kreeger, 2003), coinciding with the mating season (Seal et al., 1979). Spermatogenesis in grey wolves is also seasonal, peaking during the mating season (Kreeger, 2003). Male red wolves also show seasonal patterns in testosterone levels, with testosterone increases beginning four months before oestrus and peaking coincident with oestrus and maximum sperm production (Walker et al., 2002). In coyotes, serum testosterone levels were found to be positively correlated to testicular volume and

production of functionally intact and morphologically normal spermatozoa. In addition, coyotes show seasonal differences in testosterone, testicular volume, ejaculate volume and sperm concentration, with each of these parameters peaking during the breeding season (Minter & DeLiberti, 2008).

2.4C Maned wolves and bush dogs

Maned wolves are also seasonal breeders. However, although no seasonal pattern in testosterone was detected in male maned wolves, males were found to be aspermic outside of the breeding season (Velloso et al., 1998). Bush dogs, in contrast to most wild canid species, breed aseasonally and are occasionally polyoestrous (Porton et al., 1987). In captivity, bush dogs were found to breed almost uninterruptedly throughout the year (Porton et al., 1987), indicating that sperm production is constant throughout the year.

2.4D Red and Arctic foxes

Red foxes are seasonal breeders, with a winter breeding season in the wild between January and March (Maurel & Boissin, 1981). Red foxes show clear seasonal patterns in testosterone levels, with peaks during the breeding season (Maurel & Boissin, 1981; Andersen et al., 2001). Testicular size and spermatogenesis are also seasonal (Joffre, 1977). Similarly, Arctic foxes are seasonal breeders, with an approximately six week mating season lasting from mid March to late April (Smith et al., 1984). Arctic foxes show seasonality in androgens (androstenedione and testosterone), with peak levels during the mating season (Smith et al., 1985). Arctic foxes also show seasonal patterns in testicular weight, with testicle size peaking during the mating season (Smith et al., 1985, see Fig. 2.3).

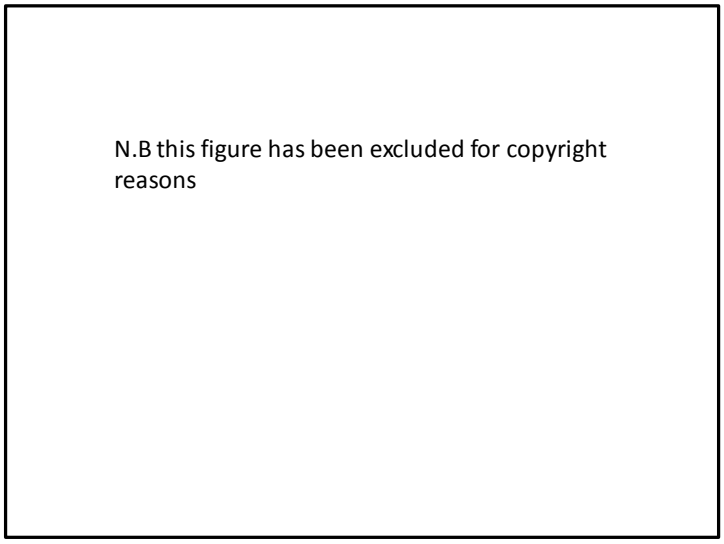

Figure 2.3: Seasonal patterns in testicular weight and plasma testosterone in Arctic foxes. Adapted from Smith et al. (1985)

2.4E African wild dogs

African wild dogs are seasonal breeders, yet in captivity, serum testosterone levels exhibited no seasonal effects, although testicular volume increased up to four fold in dogs in the summer as compared to spring, which is the main breeding season for captive African wild dogs in Australia, where the study took place (Johnston et al., 2007). Spermatogenesis was also found to be seasonally dependent (Johnston et al., 2007). Similarly, Creel et al. (1997a) also found that faecal testosterone levels did not change between mating and non mating periods in wild African wild dogs, although it should be noted that Montfort et al. (1997) did find a seasonal trend in testosterone levels in two captive males housed in North America.

The studies done by Johnston et al. (2007) and Creel et al. (1997a) on African wild dogs, and by Velloso et al. (1998) on maned wolves show that in some canid species, reproductive parameters such as testicular volume and spermatogenesis show seasonal trends, even if testosterone levels do not. This is an interesting result, as the link between testosterone and spermatogenesis has been firmly established (e.g. Hadley, 2000; Zirkin,

1998). Although studies have shown that spermatogenesis may persist when levels of testosterone are artificially lowered to a fraction of normal levels (e.g. Zirkin et al., 1989), the opposite, i.e. absence of spermatogenesis despite normal levels of testosterone, is unusual. Velloso et al. (1998) suggest their results may have been due to the fact that the focal maned wolves were not sampled for a sufficient period of time, and Johnston et al. (2007) point out that their result merits further investigation. It is possible that in these two species other androgens, such as dihydrotestosterone, play a role in spermatogenesis. Chen et al. (1994) tested the possible role of dihydrotestosterone on spermatogenesis in rats. The endogenous testosterone production in rats was artificially suppressed, but exogenous administration of dihydrotestosterone could maintain spermatogenesis. It is possible that in African wild dogs and maned wolves, other androgens play an important role in spermatogenesis, although this warrants further investigation.

2.4F Raccoon dogs

Raccoon dogs are seasonal breeders and are monoestrous (Asikainen et al., 2003). The mating season is in February and March in Western Europe (Helle & Kauhala, 1995; Rudert et al., 2010) and around between February and April in Japan (Kauhala & Saeki, 2004a). In captivity raccoon dog testosterone levels were at baseline levels from April through September, began to rise in October, and peaked in February, coincident with the mating season (Rudert et al., 2010). Testicular size and weight also increase during the mating season (Weng et al., 2006), and spermatogenesis is seasonal (Qiang et al., 2003; Weng et al., 2006).

2.5 Discussion

Several reproductive characteristics seem to be common across the Canidae. Most canid pups have a prolonged period of dependency, and cooperative breeding, including

alloparental care is common in canids (Macdonald et al., 2004). With the development of reproductive physiology techniques, including those for measuring hormones in faecal samples, new knowledge has emerged about the physiology of reproduction in canids, from both captive and wild populations. The reproductive phases and their associated hormonal changes described for domestic bitches (Jöchle & Andersen, 1976) are also found in other canid species studied to date (Table 2.2). Female canids may become pseudopregnant following a non-conceptive oestrus, and many canids have shown evidence of possible pseudopregnancy (Table 2.2). However, the hormone levels between pregnant and pseudopregnant females vary between species. In domestic dogs, serum progesterone levels of pregnant and pseudopregnant females are indistinguishable (Concannon et al., 1975), and pseudopregnant females may also show physical signs of pregnancy such as an extended abdomen (Chakraborty, 1987). However, in maned wolves, pregnant females show higher faecal progesterone levels than do pseudopregnant females (Velloso et al., 1998). Despite the fact that many canids are seasonal breeders, seasonal patterns in testosterone are not consistently found. Some species, such as coyotes (Minter & DeLiberti, 2008) do show seasonal trends in testosterone, whilst others such as African wild dogs (Johnston et al., 2007) apparently do not, despite showing seasonal trends in other reproductive parameters such as testicular volume.

The reproductive features of the Canidae studied to date reflect their life history and evolutionary adaptations. Most canids occur in places where there are distinct seasons, either the temperate summer, autumn, winter and spring seasons (e.g. grey wolves, Seal et al., 1979), or, around the equator, wet and dry seasons (e.g. Ethiopian wolves, Sillero-Zubiri et al., 1998). For this reason, seasonal reproduction, as seen in almost all canids makes adaptive sense from an evolutionary perspective, so that the birth of young can be

timed to coincide with a period when conditions are best and prey abundant. The costs of rearing young appear to be high in canids, and indeed most canids depend on alloparental care from non-breeding individuals (see Macdonald et al., 2004; Geffen et al., 1996). The adaptive function of pseudopregnancy was first suggested by Macdonald (1980), who observed pseudopregnancy and subsequent lactation in a subordinate female red fox, and suggested this served to prepare non-breeding females for their role of helpers. As pseudopregnancy can cause maternal behaviours and, in some cases, lactation (Macdonald, 1980; Chakraborty, 1987; Concannon et al., 2009), the benefits of pseudopregnancy in cooperative breeders are clear.

Although the reproductive physiology of some canid species has been studied in detail, knowledge about others, including the Ethiopian wolf, is lacking. A greater understanding of reproductive physiology is important for managing both captive and free-living canid populations (Sillero-Zubiri et al., 2004a), and it is especially vital for rare and endangered species. The endangered Ethiopian wolf is the world's rarest canid, and reproductive management initiatives including captive breeding have been recommended for the conservation of this species (Sillero-Zubiri & Macdonald, 1997). For this reason, a detailed study on Ethiopian wolf reproductive physiology was considered to be integral to the understanding and conservation of this rare and charismatic canid.

Chapter 3: Preservation of Ethiopian wolf faecal samples for hormone analysis

Abstract

Although reproductive physiology studies originally focused on domesticated or captive species, advances in hormone assay techniques have made it possible for researchers to study hormone profiles in free-living populations. However, studies in free-living populations often take place in remote environments, which means that storing and transporting samples frozen, as is recommended by most authors may be difficult. As part of a three year study on Ethiopian wolf, *Canis simensis*, reproductive physiology, a practical method of preserving Ethiopian wolf faecal samples was validated. Forty four fresh faecal samples were collected from Ethiopian wolves, and these were subdivided into three storage treatments: frozen (control), extracted in methanol and desiccated in a camping oven. The samples were then analyzed for oestradiol, progesterone, testosterone and cortisol using enzyme immunoassays. Both alternative treatments could be used to accurately predict hormone levels in control samples, although desiccated samples gave more accurate results for oestradiol and progesterone. In conclusion, desiccating Ethiopian wolf faecal samples provided a practical and reliable way of preserving the faecal samples for hormone analysis, without the need for freezing.

3.1 Introduction

Faecal samples provide a way for researchers to non-invasively study the physiology of reproduction or stress in animals, whilst avoiding the need for trapping and handling individuals. Increasingly, faecal hormone studies are carried out in wild populations. For example, faecal samples have been used to study the oestrous cycle of wild black rhinoceros, *Diceros bicornis minor* (Garnier et al., 2002), testosterone levels in wild male muriquis, *Brachyteles arachnoides* (Strier et al., 1999), and the impact of snowmobile activity on wild elk, *Cervus elaphus*, and grey wolf, *Canis lupus*, glucocorticoid levels

(Creel et al., 2002; for a review see Schwarzenberger, 2007). However, despite the advantages of using assaying hormones in faecal samples to non-invasively study wild populations, there are several challenges which are usually not encountered in studies using domesticated or captive species. Such challenges include more challenging environments, less regular sampling, and difficulties with storing samples and transporting them from the field to the laboratory.

Faecal samples must be preserved as soon as possible after collection, because bacterial degradation of hormones in faeces occurs within hours of defecation (Wasser et al., 1988). Initial faecal hormone studies focused on domesticated or captive animals, and as such, storage and transport of samples was not usually challenging. Studies in such animals would usually involve freezing the samples at -20°C upon collection of the sample (Walker et al., 2002; Wasser et al., 1995; Chapeau et al., 1993; Montfort et al., 1997; Schwarzenberger et al., 2000; Paris et al., 2002; Sanson et al., 2005). Frozen storage of faecal samples minimizes bacterial metabolism of steroids and is the method of choice under controlled laboratory conditions (Khan et al., 2002; Terio et al., 2002). However, faecal hormone studies are increasingly being carried out in wild and often remote populations of animals, and in these types of studies it is not always logistically feasible to freeze samples in the field. For this reason, several other faecal sample storage methods have been studied.

Some authors have preserved faecal samples in ethanol (Wasser et al., 1991; Wasser et al., 1996). However, storing samples in ethanol may affect the hormone concentrations in the sample. Khan et al (2002) found marked changes in faecal glucocorticoids and oestrogen in yellow baboon, *Papio cynocephalus*, faeces stored in ethanol for 180 days. Millspaugh et

al (2003) report a significant increase in elk, *Cervus elaphus*, faecal glucocorticoids after they were immersed in 90% ethanol for 6 days. In contrast, Terio et al (2002) found that concentrations of quantifiable steroid hormones (estrogens, progestagens, androgens and glucocorticoids) in cheetah, *Acinonyx jubatus*, faeces were not significantly altered by preservation in ethanol and storage at ambient temperature for at least 14 days before extraction. Other solutions have also been tested to preserve faecal samples for hormonal analysis. Millspaugh et al (2003) tested several solutions that faecal samples may be preserved in, which are those solutions allowed by the US government for shipping faecal samples from abroad into the USA. A 2% sodium hydroxide solution was tested on deer, *Odocoileus virginianus,* and elk faeces, and this was found to increase faecal glucocorticoids. A 10% formalin solution, in contrast, decreased faecal glucocorticoids. A 2% acetic acid solution was also tested, and this treatment did not influence faecal glucocorticoids in deer and elk faeces. Faecal samples may also be dissolved in an aqueous solution and stored on filter paper (Whitten et al., 1998). This method was tested successfully on humans and non-human primates by Shideler et al. (1995) who found that samples could be stored at room temperature for a year and still yield reliable results for oestrogen conjugates and progesterone metabolites.

Faecal samples can also be dried in an oven for storage. Brockman & Whitten (1996) packaged sifaka, *Propithecus verreaux,* faecal samples in foil, flattened these to increase surface area, and dried them in a Coleman® camping oven (at 55°C to 83°C for 2-3hr) within four hours of defecation. The effectiveness of faecal desiccation was evaluated in a laboratory study assessing steroid degradation over time in desiccated sifaka faeces. No significant time effects were found on faecal oestradiol and progesterone for 1, 2 and 3 weeks of storage. However, Terio et al. (2002) stored cheetah faeces in 95% ethanol and

then dried samples in a solar oven, and found that glucocorticoid and oestrogen levels were significantly lowered by this combination treatment. In contrast, progestagen metabolite concentrations were increased using this method.

The studies discussed above highlight the fact that different storage methods give different results for individual species and hormones. Because of these discrepancies, several authors recommend that storage methods are validated for each new species (Buchanan & Goldsmith, 2004; Khan et al., 2002; Terio et al., 2002). This study focused on Ethiopian wolves, a species whose reproductive physiology had not been previously studied. The populations of Ethiopian wolves studied were in the Web Valley and Sanetti Plateau of Ethiopia's Bale Mountains National Park (BMNP) in Southern Ethiopia. Although a camp with solar power was present at each site, freezing faecal samples and transporting them frozen proved to be a significant logistical challenge, and an alternative method of storing Ethiopian wolf faecal samples was needed. The aim of this study was to validate a more practical method of storing Ethiopian wolf faecal samples for subsequent hormone analysis.

3.2 Materials and Methods

3.2A Sample collection and preservation in the field

Faecal samples were collected from wolves in two packs in the BMNP's Web Valley, namely, Sodota and Darkeena packs. All 44 samples used in this study were collected within 15 minutes of defecation, mixed thoroughly by hand and preserved on ice in a cool box until return to the camp. Nineteen of the 44 samples were from adult females, 20 were from adult males and 5 samples were from adult wolves of unidentified sex. This sample size is similar to sample sizes in other faecal sample storage studies (Terio, et al., 2002, 30

samples, Millspaugh, et al., 2003, 20 samples, (Pettitt et al., 2007, 48 samples). The 44 samples were mixed thoroughly by hand and subdivided into the following three treatments:

1. Three grams of wet weight preserved at -20°C. Since immediate extraction and analysis of fresh samples was not possible in the field, and freezing is considered the ideal faecal preservation method in controlled laboratory conditions (Khan et al., 2002; Terio et al., 2002), these samples were used as a control treatment.
2. Three grams of wet weight dried in a Coleman® camper oven, at an average temperature of 100°C (kerosene heat) for an average of 1 hour, and then stored at room temperature.
3. One gram of wet weight placed in 8ml of analytical grade methanol, shaken vigorously and left overnight. The next day these samples were 'centrifuged' by tying a string to them and whirling them over my head. 1ml of supernatant was subsequently pipetted out and left to air dry. The tubes were then stored at room temperature.

All samples were stored at the camp until transport to the Veterinary University in Vienna, Austria. Frozen samples were shipped to Vienna on dry ice, the methanol extracted and oven dried samples were shipped to Vienna at room temperature.

3.2B Sample analysis

All samples were analyzed at the Veterinary University in Vienna, Austria. Samples were analyzed using enzyme immunoassay (EIA). Antibodies used and cross-reactivities are given in Tables 3.1, 3.2, 3.3 and 3.4.

Protocol

Samples were extracted by placing 0.50g of wet faeces and 0.25g of dry faeces in 4ml of 100% methanol and 1ml distilled water. Samples were vortexed for 30 minutes on a multivortex and centrifuged for 15 minutes at 2500 r.p.m. An aliquot of extract was diluted (1:10) with assay buffer (2.42g trishydroxyaminomethane, Sigma T-1503, 20 mmol/l, 17.9g NaCl, Sigma S-9625, 0.3mol/l, 1 g of bovine serum albumin, Sigma A4503,1 ml Tween 80, Sigma P-8074, dissolved up to 1 litre with double distilled water and adjusted to pH 7.5 with HCl, 1 mol/litre, then filtered through Sep-Pak® C18) and the retained steroid in the column was stored at -20°C until analysis with enzyme immunoassay. The methanol extracted samples were reconstituted with assay buffer and used for analysis.

96 well microtitre plates were coated with a solution consisting of 25µg coating antibody dissolved in 25ml coating buffer (1.59g Na_2CO_3, Sigma S-7795, and 2.93g $NaHCO_3$, Sigma S-6014, dissolved and filled up to 1 litre with double distilled water, adjusting to pH 9.6 with HCL, 1 mol/litre, then filtered through Sep-Pak® C18). Microtitre plates (F96 MaxiSorp, No. 442404, Co, Nunc, Denmark) were used, and each well was coated with 0.25 ml of diluted antibody. Plates were incubated at room temperature overnight. The next day the coating solution was discarded and each well was refilled with 0.3ml 'second' coating buffer (3.146 g Trishydroxyaminomethane, 23.3g NaCl, Sigma S-9625, 13g BSA, Sigma A-4503, 1.3g Sodium azide, Merck 106688, dissolved and added up to 1.3 litres of double distilled water, and adjusted to pH 7.5 with HCl, 1 mol/litre and filtered through Sep-Pak® C18). Plates were incubated for at least 3 hours at room temperature. Plates were then washed three times with wash buffer (0.5ml Tween 20, Merck 822185 with 2.5L double distilled water).

Assays consisted of:

-NSBs (Non Specific Binding) containing 150µl of assay buffer and 100µl of biotin labelled steroid. NSBs do not contain any primary antibody and as such give a measure of background signal.

-B0s containing 50µl of assay buffer, 100µL of primary antibody and 100µL of biotin labelled steroid. These samples contain no sample or standards and therefore provide a measure of maximum binding in the absence of competition for binding.

-Standards consisting of 50 µL of standard solutions. Standard solutions used were 1.95, 3.9, 7.8, 15.6, 31.2, 62.5, 125 and 250 pg/100µL. 50µL of antibody and 50µL of biotin labelled steroid was added to each standard.

-Samples consisting of 10µL of sample in 40µL of assay buffer. 100µL of antibody and 100µL of biotin labelled steroid was added to each sample.

Once samples, standards, NSBs and B0s had been set up and buffer, biotin labelled steroid and antibody had been added where necessary, plates were shaken briefly and incubated overnight at 4°C. After this incubation plates were washed four times with washing solution. 250µL of enzyme solution (30ml assay buffer, 1µL Streptavidin-POD-conjugate, 0.5 U; Noehringer 1089153, 500 U, mixed on magnetic stirrer a few minutes before use) was added to each well. Plates were covered and incubated for 45 minutes at 4°C on a multiplate shaker. Plates were washed again four times with washing solution, and 250 µL of substrate solution was added to each well (30 ml assay buffer, 0.5ml 3,3',5,5'-Tetramethylbenzedine, 0.4% and 1 ml H_2O_2, 0.6% 0.3 ml H_2O_2 [35%, Merck 108600] + 17.5ml double distilled water in substrate buffer: 1.36 g Sodium acetate, Merck 6267, 10 mmol/l dissolved and filled up to 1 litre, adjusted to pH 5.0 with 5% citric acid, Merck

100244). Plates were incubated with substrate solution for 45 minutes at 4°C. After this incubation, 50µL of Stop solution (900ml double distilled water and 100ml H_2SO_4 [95-97% Merck 100731]) was added. Plates were read with an automatic multiplate reader using a measuring filter of 450nm.

Table 3.1: Oestradiol antibody cross-reactivities (Palme & Möstl, 1993)

Oestrogen antibody cross reactivities	**sheep-anti17β-oetradiol-17HS antibody**
1,3,5(10)-Estratrien-3-ol-17-one (Oestrone)	100.00%
1,3,5(10)-Estratrien-3-17α-diol (Oestradiol 17α)	19.00%
1,3,5(10)-Estratrien-3-17β-diol (Oestradiol 17β)	70.00%
1,3,5(10),7-Estratraen-3-16a-17β-triol (Oestriol)	129.00%
1,3,5,(10)Estratetraen3,17β-diol	20.00%
1,3,5(10),7-Estratraen-3-ol-17-one	87.00%
1,3,5(10),6,8Estrapentaen-3,17β-diol	0.80%
1,3,5(10),6,8Estrapentaen-3,-ol-17-one	1.10%
1,3,5(10)Estratrien-3-ol-17-on-3-sulfat	<0.10%
1,3,5(10)Estratrien-3-ol-17-on-3-gluconoride	<0.10%

Table 3.2: Testosterone antibody cross-reactivities (Palme & Möstl, 1993)

Testosterone antibody cross reactivity	**Anti-testosterone -3-CMO-BSA**
4-Androsten 17β-ol-3-one (testosterone)	100.00%
5α-androstan-17β-ol-3-one (5α-dihydrotestosterone)	23.70%
5β-androstan-17β-ol-3-one (5β-dihydrotestosterone)	12.30%
4-Androsten 3β, 17βdiol	7.60%
5α-Androsten 3α, 17βdiol	5.50%
5α-Androsten 3β, 17βdiol	1.30%
5β-Androsten 3α, 17βdiol	1.10%
4-Androsten 17α-ol-3-one (epitestosterone)	<0.10%
5α-Androstan-3α-ol-17-one (androsterone)	<0.10%
5α-Androstan-17α-ol-3-one	<0.10%

Table 3.3: Progesterone antibody cross-reactivities (Schwarzenberger et al., 1996)

Progesterone antibody cross reactivity	**5β-pregnane-3a-ol-20-on3e HS:BSA**
4-pregnen-	
3,20 dione (progesterone)	100.00%
3β-ol-20-one	68.00%
3α-ol-20-one	20.00%
20α-ol-3-one	<0.10%
20β-ol-3-one	<0.10%
5α-pregnane	
3α-ol-20-one	8.00%
3β-ol-20-one	102.00%
3,20-dione	75.00%
3α,20α-diol	<0.10%
3α,20β-diol	<0.10%
5β-pregnane	
3α-ol-20-one	20.00%
3β-ol-20-one	36.00%
3,20-dione	151.00%
3α,20α-diol	<0.10%
3α,20β-diol	<0.10%
5-pregnen	11.00%
Cortisol	<0.10%
Estrone	<0.10%

Table 3.4: Cortisol antibody cross-reactivities. Note that two antibodies were used.(Palme & Möstl, 1996)

Cortisol antibody cross reactivity	**11-oxoetiocholanolone**	**cortisol-3-CMO**
4-pregnene-		
11β,21-diol-3,20-dione	<0.10%	6.20%
11β,17a,21-triol-3,20-dione	<0.10%	100.00%
5α-pregnane		
11β,17a,21-triol-3,20-dione	<0.10%	4.60%
3a,11β,17a,21-tetrol-21	<0.10%	0.80%
5β-pregnane		
3a,11β,17a,21-tetrol-20-one	<0.10%	0.10%
17a,21-diol-3,11,20-trione	<0.10%	<0.10%
3a,11β,21-triol-20-one	<0.10%	<0.10%
3a,17a,21-triol-11,20-dione	<0.10%	<0.10%
3a,17a,20a,21-tetrol-11-one	<0.10%	<0.10%
3a,11β,17a,20a,21-pentol	<0.10%	<0.10%
5a-Androstane		
3a-ol-11,17-dione	5.60%	<0.10%
3β-ol-11,17-dione	6.70%	<0.10%
3,11,17 trione	14.70%	<0.10%
5β-Androstane		
3a,11β-diol-17-one	6.00%	<0.10%
3a-ol-11,17-dione	100.00%	<0.10%
3,11,17 trione	84.00%	<0.10%

3.2C Data analysis

Results are given as ng/g wet or dry faeces. No effort was made to correct for the difference in water content in frozen/extracted and desiccated faeces, as we were not interested in the absolute concentrations of hormones in each sample but in the relative proportions between samples, that is, to see if the treated samples accurately reflected the controls. Differences between treatments were assessed using paired T tests. Regression analysis was used to see how well the hormone levels for the frozen treatment could be predicted by the two treatments, namely desiccation and methanol extraction (Grafen & Hails, 2002). All statistical analysis was done with Minitab® statistical software.

3.3 Results

Progesterone

Frozen samples yielded progesterone values between 2.2 and 3601.7 ng/g wet faeces. Oven desiccated samples yielded progesterone values between 99.9 and 4687.3 ng/g dry faeces. Methanol extracted samples yielded progesterone values between 24.1 and 675.1 ng/g wet faeces (Fig. 3.1). As compared to the frozen (control) samples, desiccated samples gave higher values of progesterone. The difference between the frozen and desiccated samples was significant (paired T test, $p=0.001$). Methanol extracted samples generally gave lower values of progesterone, although this difference was not significant (paired T test, $p=0.303$).

Desiccated samples could be used to accurately predict progesterone levels in the frozen (control) samples (regression, $F_{1,22}=61.66$, R^2 adjusted=72.5%, $p<0.001$), especially when only those samples known to be from females (i.e. excluding the five samples from unidentified wolves) were considered (regression, $F_{1,17}=269.83$, R^2 adjusted=93.7%, $p<0.001$). Methanol extracted samples were slightly less predictive than desiccated

samples (regression, $F_{1,22}$=45.87, R^2 adjusted=66.1%, p<0.001), although this method was more predictive when only those samples known to be from females (i.e. excluding the five samples from unidentified wolves) were considered (regression, $F_{1,17}$=46.26, R^2 adjusted=71.5%, p<0.001).

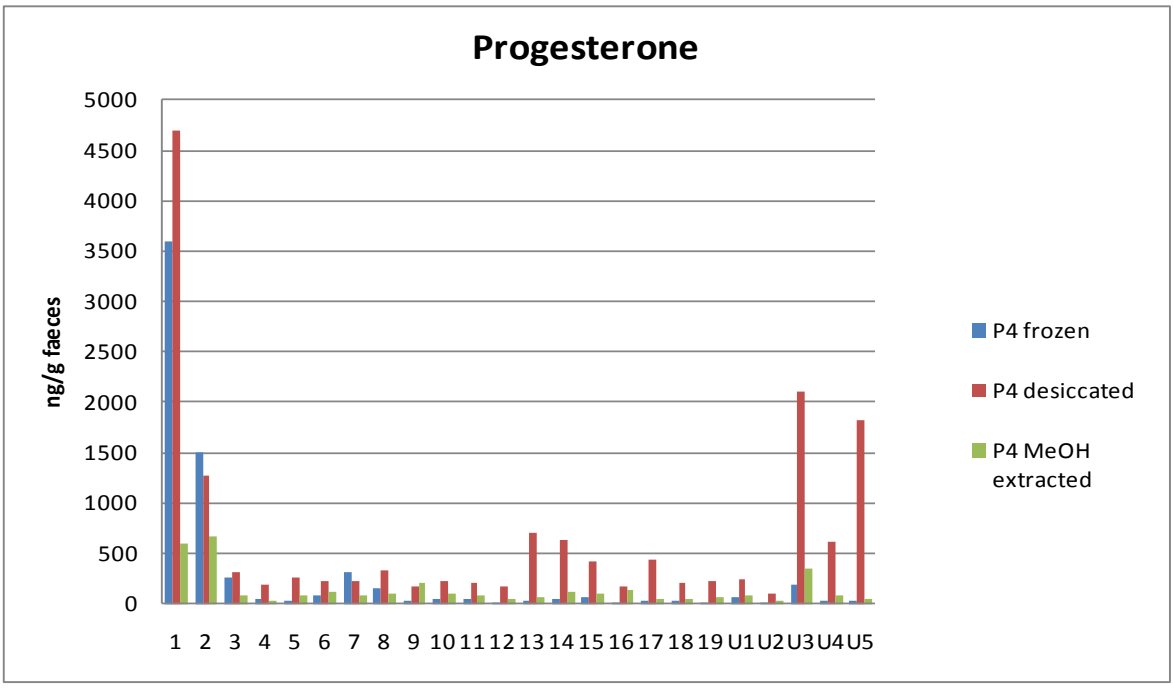

Figure 3.1: Progesterone (P4) measured in faeces of 24 Ethiopian wolf faecal samples (19 samples from females, 5 samples from wolves of unidentified sex) preserved three different ways (frozen, desiccated and methanol extracted)

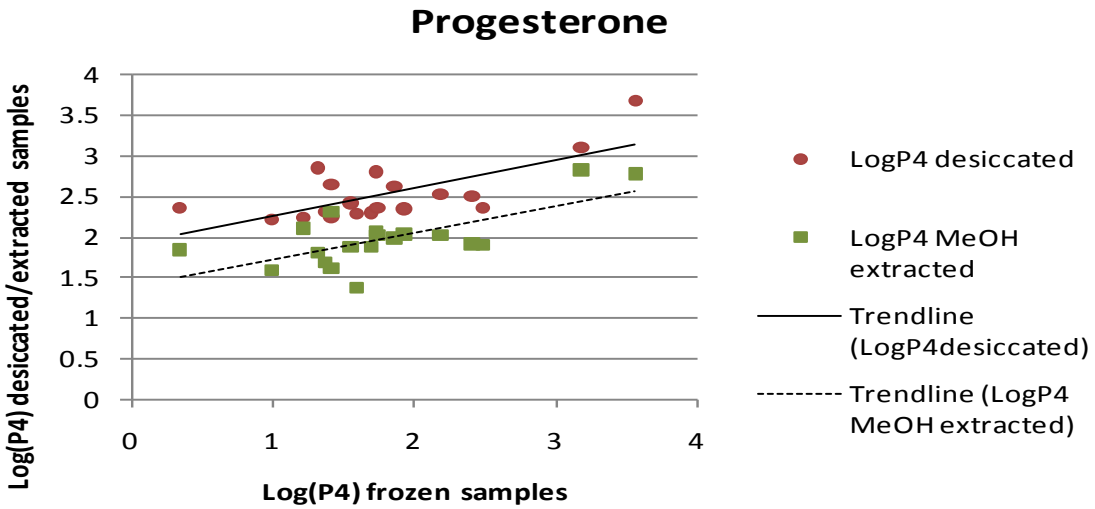

Figure 3.2: Log(Progesterone) in desiccated and methanol extracted samples (Y-axis) as compared to Log(Progesterone) in frozen samples (X-axis). Note: figure includes data from 19 samples from females.

Oestradiol

Frozen samples yielded oestradiol values between 0.38 and 64.8 ng/g wet faeces. Desiccated samples yielded oestradiol values between 4.1 and 69.2 ng/g dry faeces. Methanol extracted samples yielded oestradiol values between 0.12 and 5.3 ng/g wet faeces (Fig. 3.3). As with progesterone, desiccated samples generally yielded higher values than frozen samples, and this difference was significant (paired T test, $p=0.002$). Methanol extracted samples yielded significantly lower values than frozen samples (paired T test, $p<0.001$).

Desiccated samples could be used to accurately predict the oestradiol levels in the frozen (control) samples (regression, $F_{1,22}=32.53$, R^2 adjusted=57.8%, $p<0.001$), especially when only those samples known to be from females (i.e. excluding the five samples from unidentified wolves) were considered (regression, $F_{1,17}=35.98$, R^2 adjusted=67.3%, $p<0.001$). Methanol extracted samples were less accurate than desiccated samples (regression, $F_{1,22}=12.83$, R^2 adjusted=34%, $p=0.002$).

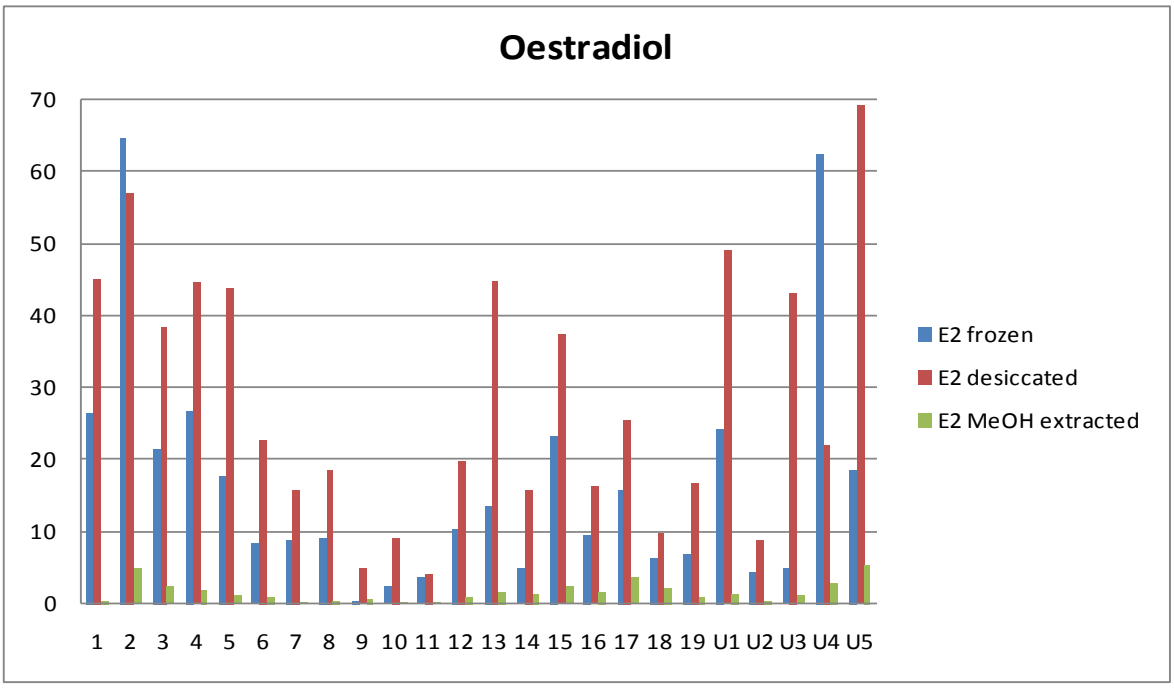

Figure 3.3: Oestradiol (E2) measured in faeces of 24 Ethiopian wolf faecal samples (19 samples from females, 5 samples from wolves of unidentified sex) preserved three different ways (frozen, desiccated and methanol extracted)

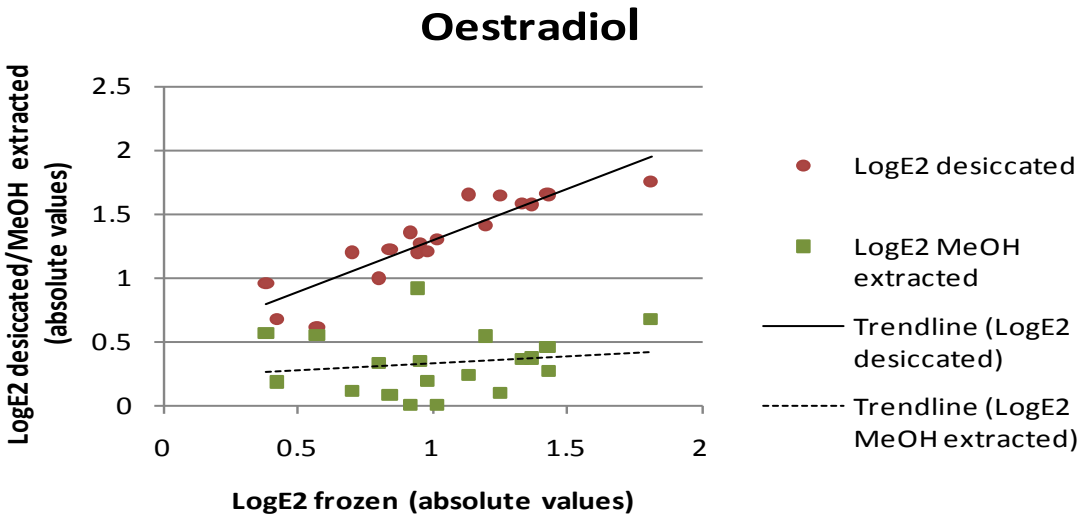

Figure 3.4: Log(Oestradiol) in desiccated and methanol extracted samples (Y-axis) as compared to Log(Oestradiol) in frozen samples (X-axis). Note: figure includes data from 19 samples from females.

Testosterone

Frozen samples yielded testosterone values between 2.53 and 133.8 ng/g wet faeces. Desiccated samples yielded testosterone values between 0.88 and 319.5 ng/g dry faeces. Methanol extracted samples yielded values between 1.8 and 29.0 ng/g wet faeces (Fig. 3.5). Desiccated samples yielded significantly higher values than frozen samples (paired T test, $p=0.007$), and methanol extracted samples yielded significantly lower values than frozen samples (paired T test, $p=0.001$).

Although less predictive than for progesterone and oestradiol, desiccated samples could be used to accurately predict the testosterone levels in the frozen (control) samples (regression, $F_{1,23}=17.51$, R^2 adjusted=46.5%, $p=0.001$). Methanol extracted samples were more accurate (regression, $F_{1,23}=89.51$, R^2 adjusted=82.3%, $p<0.001$).

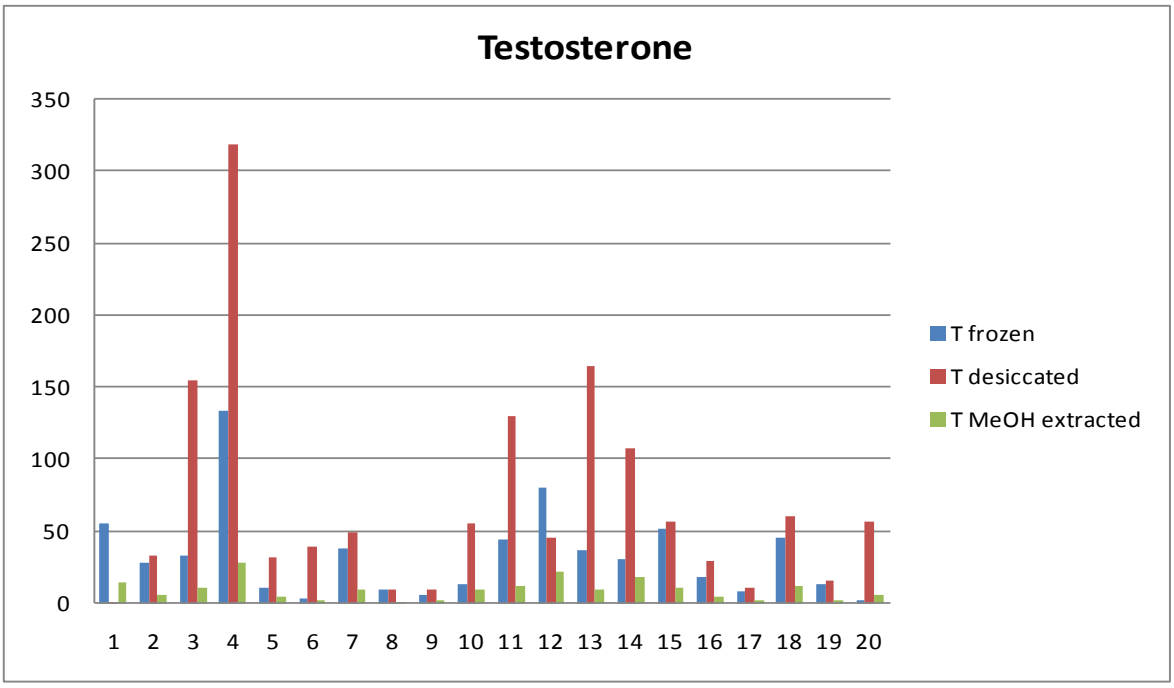

Figure 3.5: Testosterone (T) measured in faeces of 20 faecal samples from male Ethiopian wolves preserved three different ways (frozen, desiccated and methanol extracted). Note that the five samples of wolves of unidentified sex that were extracted in methanol were not assayed for T and hence are not included in this figure.

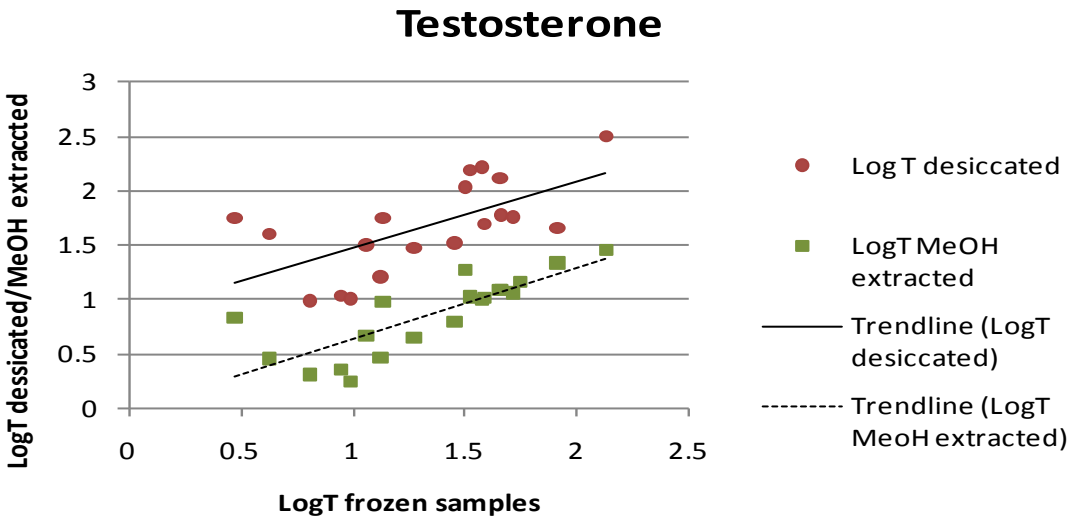

Figure 3.6: Log(Testosterone) in desiccated and methanol extracted samples (Y-axis) as compared to Log(Testosterone) in frozen samples (X-axis). Note: figure includes data from 20 samples from males.

Cortisol

Frozen samples yielded cortisol values between 1.26 to 65.4 ng/g wet faeces. Desiccated samples yielded cortisol values between 0.22 and 113.8 ng/g dry samples. Methanol

extracted samples yielded cortisol values between 0.81 and 31.3 ng/g wet faeces (Figs. 3.7, 3.9). Desiccated samples yielded significantly higher values than controls (paired T test, p=0.003) and methanol extracted samples yielded significantly lower values than frozen samples (paired T test, p=0.008).

Desiccated samples corresponded less well with controls than for progesterone, oestradiol and testosterone (regression, $F_{1,41}$=13.03, R^2 adjusted=21.9%, p=0.001). Methanol extracted samples gave marginally better results (regression, $F_{1,41}$=15.12, R^2 adjusted=24.7%, p<0.001). However, when the samples were split between samples from males and females, results showed that desiccated samples could be used to accurately predict the cortisol levels in the frozen (control) samples from females (regression, $F_{1,17}$=55.92, R^2 adjusted=75.3%, p<0.001), although desiccated samples did not accurately predict cortisol level in male samples (regression, $F_{1,22}$=2.37, R^2 adjusted=6.7%, p=0.141). Methanol extracted samples were less reliable for females (regression, $F_{1,17}$=8.60, R^2 adjusted=29.7%, p=0.009) but more reliable for males (regression, $F_{1,22}$=18.41, R^2 adjusted=47.8%, p<0.001).

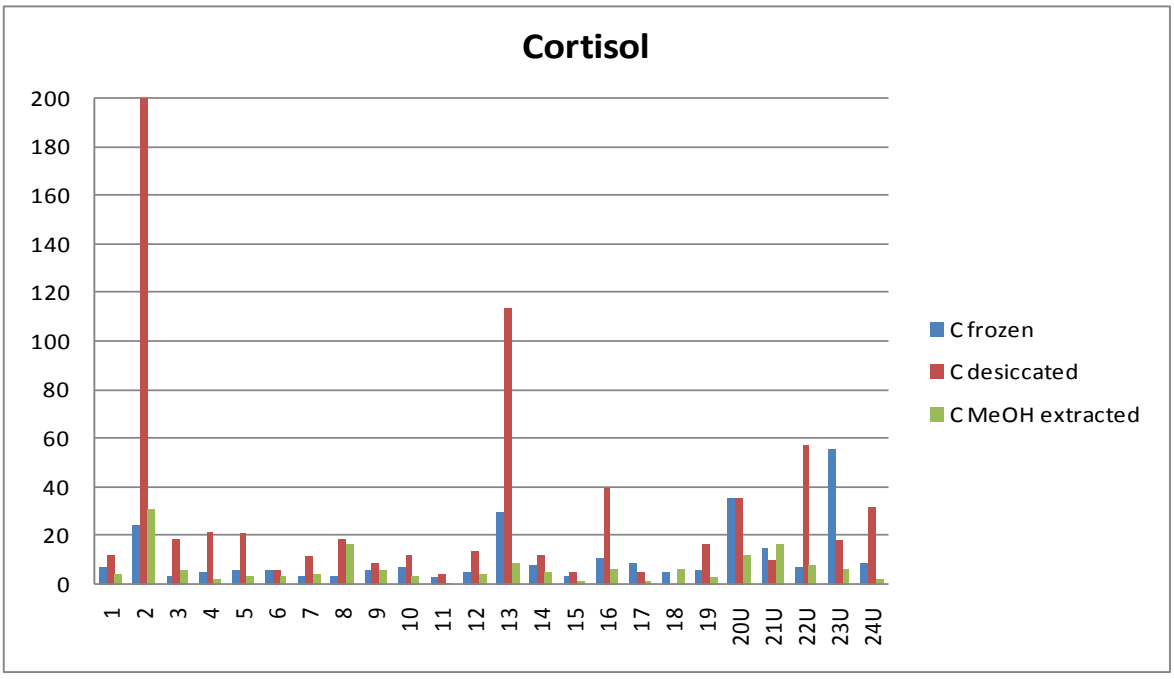

Figure 3.7: Cortisol measured in 24 faecal samples from Ethiopian wolves (19 female, 5 unknown sex) preserved three different ways (frozen, desiccated and methanol extracted)

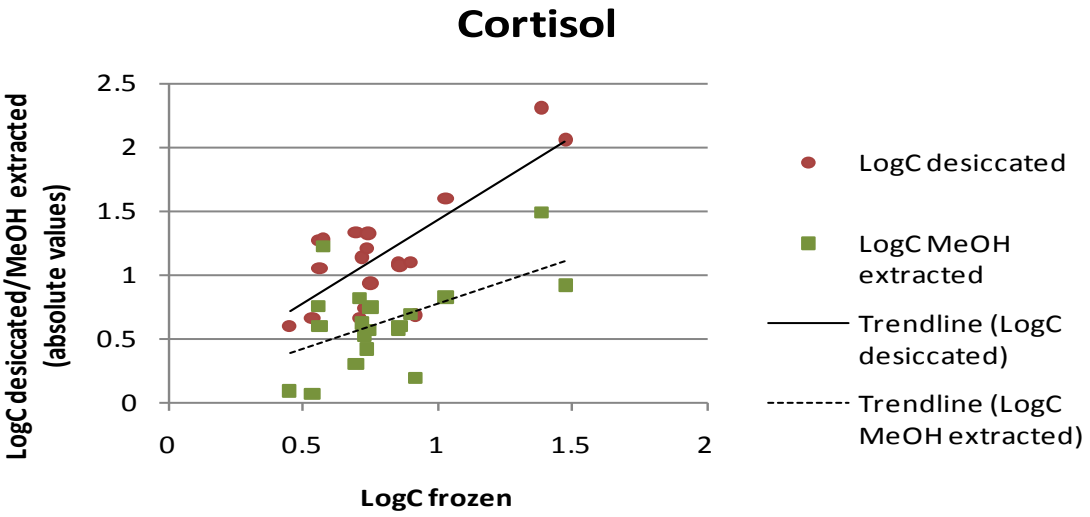

Figure 3.8: Log(Cortisol) in desiccated and methanol extracted samples (Y-axis) as compared to Log(Cortisol) in frozen samples (X-axis). Note: figure includes data from 19 samples from females.

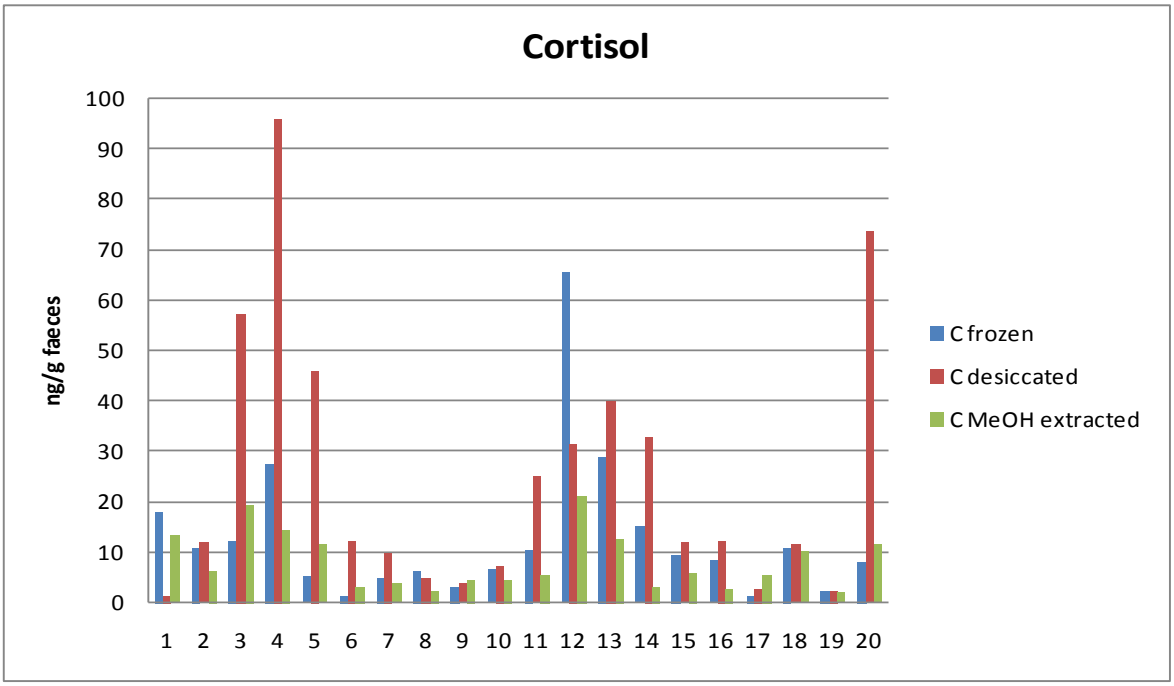

Figure 3.9: Cortisol measured in 20 faecal samples from male Ethiopian wolves preserved three different ways (frozen, desiccated and methanol extracted)

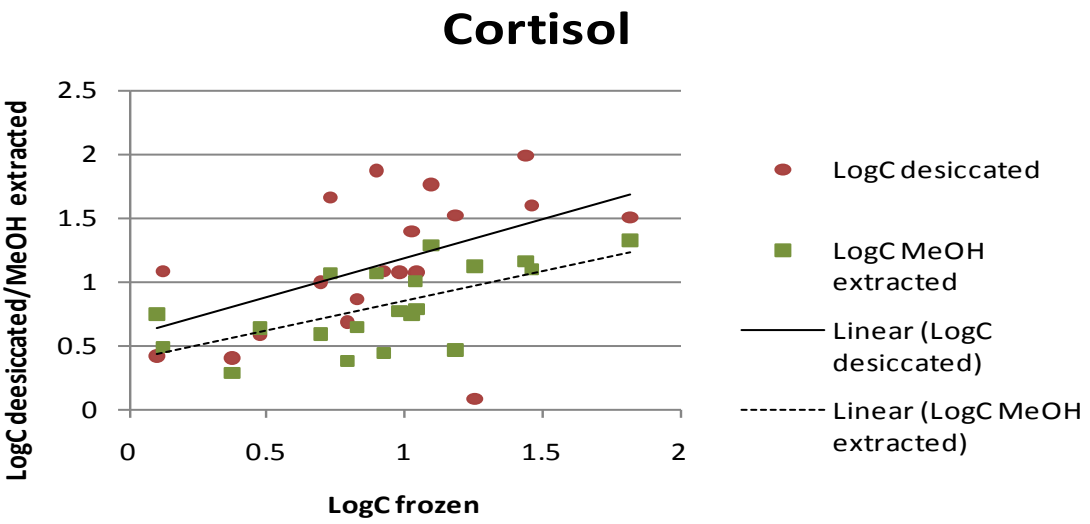

Figure 3.10: Log(Cortisol) in desiccated and methanol extracted samples (Y-axis) as compared to Log(Cortisol) in frozen samples (X-axis). Note: figure includes data from 20 samples from males.

3.4 Discussion

Because of the logistical challenges of storing and transporting frozen samples, a single alternative method for preserving the faecal samples was needed. Although the three storage methods (frozen, desiccated and methanol extracted) yielded significantly different values for each of the hormones, the overall concentrations of progesterone, oestradiol, testosterone and cortisol corresponded significantly between the treatments and controls ($p<0.05$). The differences between the desiccated and frozen samples are most probably related to the water content in the frozen samples. The water content of human faeces is estimated to be between 50-75% (see Stephen & Cummings, 1980). This means hormone concentrations would be between 2-4 times higher per gram of dry faeces as compared to per gram of wet faeces. Although the water content of the Ethiopian wolf faecal samples was not directly determined, the dried samples yielded hormone concentrations that were on average 2.2 times higher than those in frozen samples, which is consistent with the range estimated for humans. The methanol extracted samples yielded hormone concentrations that were much lower than those in frozen samples. This is probably

because the extraction method in the field was less thorough. For instance, the extracted samples were shaken in the field by hand and whirled on a string whereas the frozen samples were shaken on a multivortex for 30 minutes and centrifuged at 2500 r.p.m for 15 minutes. Nevertheless, the results indicate that desiccated samples could be used to reliably measure oestradiol, progesterone, cortisol (in females), and, to a somewhat lesser extent, testosterone in Ethiopian wolf faecal samples. The results for cortisol varied greatly when the sample set was split between samples collected from males and females. Sex differences in excreted hormones have also been observed in cats (Schatz and Palme 2001) and ponies and pigs (Palme et al. 1996), and different excretions of cortisol between males and females may explain the pattern found here.

Although studies on faecal sample storage usually compare control (frozen) samples with treatments and test for differences between them (e.g. Millspaugh et al., 2003), this study does not focus on differences between absolute concentrations of hormones but between relative concentrations or patterns. Since longitudinal studies of hormone secretion (including those described in chapters four and five) usually focus on seasonal patterns, or on differences between dominant and subordinate animals, the absolute values of hormones are not relevant, but the relative values between seasons and/or individuals are. Therefore, although the absolute values of our treated samples differed significantly from the controls, the fact that they corresponded significantly to the controls meant that the treated samples could be used to study patterns in hormone levels.

Desiccated samples better predicted the control (frozen) samples than methanol extracted samples for progesterone and oestradiol, and were more predictive for cortisol in samples of females. In addition, desiccating samples was more practical than extracting samples in

the field, as methanol can be difficult to source in Ethiopia. For this reason desiccating samples in a Coleman® camper oven was the method selected for the remainder of the study.

Chapter 4: Sex, suppression and pseudopregnancy in female Ethiopian wolves[1]

[1] A version of this chapter has been prepared for submission to Hormones and Behavior as: van Kesteren et al, The physiology of cooperative breeding in a rare social canid; sex, suppression and pseudopregnancy in female Ethiopian wolves

Abstract

The reproductive physiology of the female Ethiopian wolf, the world's rarest canid, was assessed non-invasively. Faecal samples and behavioural observations were collected from fourteen dominant and nine subordinate female wolves in Ethiopia's Bale Mountains National Park. The collected samples were analyzed for oestradiol, progesterone and cortisol using enzyme immunoassays (EIA) and radio immunoassays (RIA). All fourteen dominant females showed oestrus behaviour including mating and/or an oestradiol peak during the annual mating season. In contrast, none of the subordinate females showed oestrous behaviour or an oestradiol peak during the annual mating season, indicating they were hormonally reproductively suppressed. Three subordinate females, however, came into oestrus outside the annual mating season. Ten out of 13 pregnant females showed increases in faecal progesterone during their pregnancy. Seven subordinate females also showed increased faecal progesterone during the time their dominant female was pregnant. Two subordinate females also allosuckled the pups. These results suggest that subordinate females may ovulate outside of the annual mating season and become pseudopregnant. There were no significant differences in cortisol levels between dominant and subordinate females, suggesting that reproductive suppression in Ethiopian wolves is not mediated mainly through stress or stress hormones.

4.1 Introduction

Less than 500 adult Ethiopian wolves survive (Marino et al., 2006). Several conservation measures have been suggested for this endangered canid, including semen and egg cell banking and captive breeding (Sillero-Zubiri & Macdonald, 1997). If these conservation measures are to be implemented, a more detailed understanding of Ethiopian wolf reproduction is needed. Previous behavioural and genetic work has provided us with some

insight into Ethiopian wolf reproduction (Chapter 1), but the reproductive physiology of this species has not been studied before. There are no Ethiopian wolves in captivity (Sillero-Zubiri & Macdonald, 1997), but recent advances in reproductive technologies have made it possible to study the reproductive physiology of wild populations non-invasively through assaying hormones extracted from faecal or urine samples (Whitten et al., 1998). These non-invasive techniques were used to study the reproductive physiology of Ethiopian wolves in Bale Mountains National Park, Southern Ethiopia.

Ethiopian wolves are endemic to the Ethiopian highlands (Sillero-Zubiri & Gottelli, 1994), where they have become specialized in hunting the many, often also endemic, rodents that occur in their Afroalpine habitats (Sillero-Zubiri & Gottelli, 1995a). Ethiopian wolves live in family packs that usually consist of one to three adult females, one to seven adult males, and one to six yearlings and/or pups (Sillero-Zubiri et al., 1996a). Packs are territorial, and all pack members help to patrol and defend territory boundaries (Sillero-Zubiri & Macdonald, 1998). Within a pack there is a dominance hierarchy among both males and females (Sillero-Zubiri & Gottelli, 1994).

Ethiopian wolves are cooperative, seasonal breeders, and females give birth once a year, at the end of the rainy season (October-January, Sillero-Zubiri et al., 1998). Gestation lasts about 60 days and litters usually consist of one to six pups (Sillero-Zubiri & Gottelli, 1994). Males and females reach sexual maturity in their second year (Sillero-Zubiri & Gottelli, 1994). All pack members help rear the pups by guarding the den and regurgitating prey to the pups (Sillero-Zubiri & Gottelli, 1994; Sillero-Zubiri et al., 2004b). In addition, Sillero-Zubiri et al. (1996a) found that in 8 out of 18 dens watched, a subordinate female allosuckled the pups. Usually only the dominant female in a pack breeds, but one study

found that 3 of 12 observed mating events involved subordinate females (Sillero-Zubiri et al., 1996a), and although extremely rare, subordinate females may have pups. One of the three subordinate females seen mating by Sillero-Zubiri et al. (1996) gave birth but failed to rear pups, and Randall et al. (2007a) found that one of 48 pups could be assigned to a subordinate female. Although within a pack the dominant female mates only with the dominant male, extra pair copulations between the dominant female of a pack and males from different packs account for 70% of matings (Sillero-Zubiri et al., 1996a). These studies show that subordinate males and at least some subordinate females may exhibit normal reproductive behaviour, and multiple paternity implies that subordinate males are fertile. However, Sillero-Zubiri (1994) observed no overt aggression between male wolves during the breeding season, and males other than the dominant female's consort were excluded from the female's immediate vicinity through mild threats.

Although some aspects of Ethiopian wolf reproduction have been studied before using behavioural observations and molecular genetics (Randall et al., 2007; Sillero-Zubiri et al., 1996a; Sillero-Zubiri et al., 1998; Tallents, 2007), nothing is known about this species' reproductive physiology. This chapter aims to assess the reproductive physiology of female Ethiopian wolves for the first time. Specifically, the main research questions for this chapter are the following:

1. Are there any seasonal trends in oestradiol levels in dominant females?

   As Ethiopian wolves have only one mating season per year (Sillero-Zubiri et al., 1998), and oestradiol is associated with oestrus in other canids (Chapter 2) I would expect that dominant, breeding females will show increases in oestradiol during the mating season, but would expect that oestradiol levels at other times of the year will be low.

2. Are there any seasonal trends in progesterone levels in dominant, breeding females?

   Dominant females usually become pregnant and give birth once a year (Sillero-Zubiri et al., 1998). Since progesterone is associated with pregnancy in other canids (Chapter 2), I would expect that pregnant females show an increase in progesterone levels during pregnancy.

3. Are there differences between the oestradiol and progesterone levels of dominant and subordinate females during the mating and breeding season?

   Subordinate female Ethiopian wolves generally do not mate or breed (Sillero-Zubiri et al., 1996a, this study). I hypothesized that this is because most subordinate females, unlike dominant females, do not come into oestrus during the annual mating season. However, subordinate females do sometimes show signs of pseudopregnancy including appearing pregnant (pers. observation) and allosuckling the pups (Sillero-Zubiri et al., 1996a). As pseudopregnancy in other canids is the result of an infertile oestrus (e.g. Chakraborty, 1987), this suggests that some subordinate females do come into oestrus. We therefore predict that some subordinate females will come into oestrus and become pseudopregnant, and therefore also show increased levels of progesterone during the time that their dominant female is pregnant.

4. Are there differences in cortisol levels between dominant and subordinate females, and do these reflect patterns of aggression?

Ethiopian wolves generally show low levels of intra-pack aggression (Sillero-Zubiri, 1994, personal observation), although aggressive interactions related to territory defence are common (Sillero-Zubiri & Macdonald, 1998). It is possible that dominant females have to act aggressively more often to maintain their dominant status, which may be stressful (Creel, 2001). Several studies in other communal breeders in the wild found that dominants have higher cortisol levels than subordinates (e.g. Creel et al., 1997a; Sands & Creel, 2004). For this reason, I predicted that dominant females will be more aggressive than subordinates, and have higher cortisol levels.

4.2 Materials and Methods

4.2A Study population

Nine focal packs were selected based on the presence of dominant breeding females and subordinate females, and presence of ear tagged (and thus easily identifiable) wolves. Over the course of three years, 23 female Ethiopian wolves (14 dominant and 9 subordinate) from six packs in the Web Valley (Addaa, Darkeena, Kotera, Megity, Mulamo and Sodota packs, Figs. 4.1 and 4.2) and from three packs in the Sanetti Plateau (BBC, Dumal, and Nyala packs, Fig. 4.2) were studied (Table 4.1). All study females were at least 22 months of age when they were first included in the study. This is the age at which Ethiopian wolf females first start to show reproductive behaviour (Sillero-Zubiri & Gottelli, 1994), even though at this age females are usually subordinate, and thus unlikely to have the opportunity to breed (C. Sillero-Zubiri, pers. comm.). Study females were categorized according to pack years, so that, for example, SOD02, who was included for three consecutive years, is listed in Table 4.1 three times.

Table 4.1: Overview of the female Ethiopian wolves included in this study. Each individual is assigned a unique code consisting of the individual's natal pack and a number.

Location	Pack	ID	Status	Notes
2007				
Web Valley	Addaa	SOD06	Dominant	Split from Sodota pack
Web Valley	Darkeena	DAR02	Dominant	
Web Valley	Sodota	SOD02	Dominant	
2008				
Web Valley	Addaa	SOD06	Dominant	Died in the 2008-09 rabies epizootic
Web Valley	Darkeena	DAR02	Dominant	Died in the 2008-09 rabies epizootic
Web Valley	Darkeena	DAR10	Subordinate	Died in the 2008-09 rabies epizootic
Web Valley	Darkeena	DAR12	Subordinate	Died in the 2008-09 rabies epizootic
Web Valley	Darkeena	DAR14	Subordinate	Died in the 2008-09 rabies epizootic
Web Valley	Kotera	KOT30	Dominant	Died in the 2008-09 rabies epizootic
Web Valley	Megity	MEG02	Dominant	
Web Valley	Megity	MEG06	Subordinate	
Web Valley	Mulamo	DAR06	Dominant	
Web Valley	Mulamo	DAR08	Subordinate	Died in the 2008-09 rabies epizootic
Web Valley	Sodota	SOD02	Dominant	
2009				
Web Valley	Megity	MEG06	Dominant	Became dominant after MEG02 dispersed
Web Valley	Sodota	SOD02	Dominant	
Web Valley	Sodota	SOD04	Subordinate	
Sanetti Plateau	BBC	BBC32	Dominant	
Sanetti Plateau	BBC	BBC42	Subordinate	
Sanetti Plateau	Dumal	DUM02	Dominant	
Sanetti Plateau	Dumal	DUM04	Subordinate	
Sanetti Plateau	Nyala	NYA36	Dominant	
Sanetti Plateau	Nyala	NYA32	Subordinate	

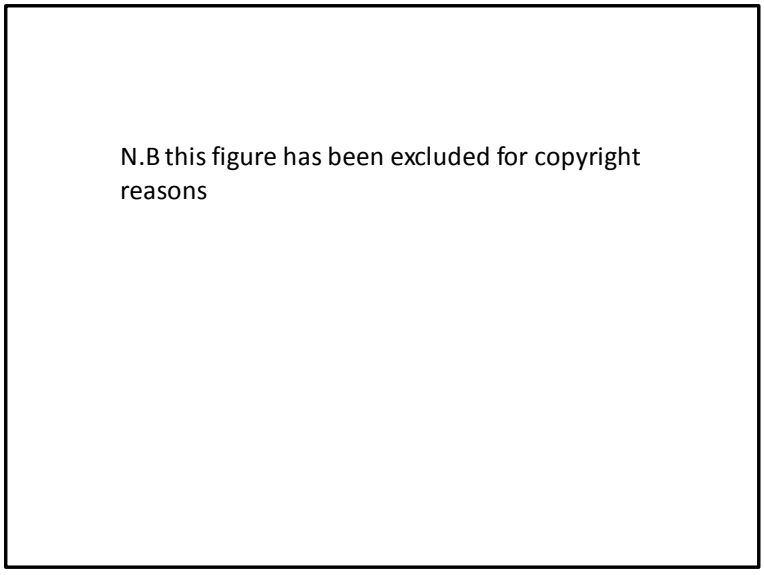

Figure 4.1: Schematic representation of packs included in the 2007-08 field season (A) and in the 2008-09 field season (B). Adult composition (males and females) in each pack is represented by symbols. Wolves who died in the 2008-09 rabies epizootic are represented as crossed out symbols. Map adapted from Randall (2006).

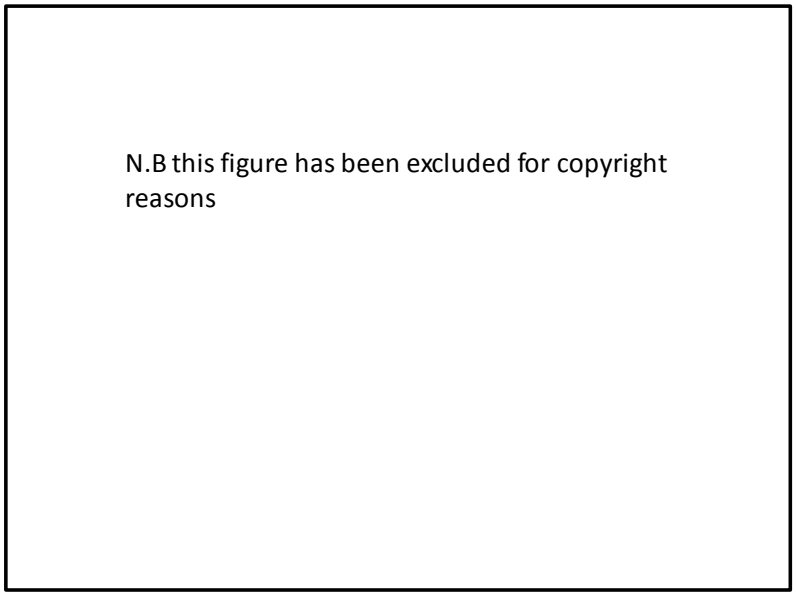

Figure 4.2: Schematic representation of packs included in the 2009-2010 field season. Adult males and females in the packs are represented by symbols. One male in Dumal pack was found dead and this is indicated by a crossed out symbol. Map adapted from Randall (2006).

4.2B Field methods

For a full description of the field methods see Chapter 1, section 1.9. Briefly, wolves were followed on foot or horseback and behavioural observations (including date and time, wolf age, sex and ID, and behavioural observations) were recorded every 15 minutes. Faecal samples were collected within minutes of defecation and stored in a cooler box on ice until return at the camp. During the 2007-08 field season, 3 grams of wet sample was stored at -20°C and then shipped to Edinburgh, United Kingdom (via Vienna, Austria), on dry ice. During the 2008-2009 and 2009-2010 field seasons, 3 and 4 grams of wet sample respectively were dried in a Coleman® Camper Oven at an average temperature of 100°C (kerosene heat) for one hour, and then stored and shipped at room temperature.

4.2C Laboratory methods

The laboratory methods are described in detail in Chapter 1, sections 1.10-1.13. Briefly, samples were extracted by manually grinding 0.50g of wet (frozen) or 0.20g of dry (desiccated) sample with 4ml analytical grade methanol and 0.50ml double distilled water,

vortexing at 1400 r.p.m, centrifuging at 2500 r.p.m, repeating the extraction process, and mixing the two supernatants. 0.50ml of supernatant was dried under mild heat and nitrogen, and reconstituted in PGBS assay buffer. Samples were analyzed for progesterone and cortisol using radio immunoassays, and for oestradiol using enzyme immunoassays.

A total of 819 faecal samples from female Ethiopian wolves were used for this study, of which 101 were stored frozen and 718 samples were desiccated and stored at room temperature. All samples from all females were analyzed for progesterone and oestradiol, and a subset of 250 samples was analyzed for cortisol.

4.2D Data analysis

Hormone concentrations are expressed as nanograms of hormones per gram of wet faeces (ng/g) for frozen samples, and as ng/g dry faeces for desiccated samples. Since desiccated samples were found to yield higher absolute values of progesterone, oestradiol and cortisol per gram of faeces (Chapter 3), no comparisons were made between absolute values of frozen and dried samples, and values for frozen and dried samples were not combined.

We were interested in seasonal patterns of progesterone and oestradiol, but absolute hormone levels varied greatly amongst individual females. For this reason, oestradiol and progesterone levels were calculated as a proportion of baseline values. Baseline values were calculated by averaging pre-oestrus values (excluding any extreme outliers). This allowed comparison between individual females, and between frozen and desiccated samples. As we did not expect seasonal patterns in cortisol (Romero, 2002), and were interested in absolute values of cortisol in dominant and subordinate females, cortisol values were left as ng/g faeces.

Thirteen of the 14 dominant females showed a clear increase in oestradiol levels around the time they started showing oestrus behaviour, but this increase differed between females. For example, female MEG02 showed a more than 9-fold increase in oestradiol from baseline levels (551.0 ng/g to 5183 ng/g dry faeces, Table 4.5), whereas female SOD02 in 2007 showed a 2.6 fold increase in oestradiol from baseline levels (241 ng/g to 623 ng/g wet faeces, Table 4.5). A 2.6 fold increase in oestradiol around the time of oestrus was the lowest observed clear increase, seen in a female who was confirmed to be in oestrus through observed mating, and subsequent pregnancy and birth, and who did not show greater oestradiol peaks at other points during the field season. This value was therefore taken as the minimum threshold to count as a peak.

Over the three years and in the two locations, the timing of the breeding season varied considerably. The 2008-09 breeding season, with mating in November and pups born in late January, was the latest ever recorded breeding season in Web Valley (Sillero-Zubiri et al., 1998). In order to compare different years and locations, calendar dates were converted to 'oestrus dates.' Oestrus dates were calculated using the first recorded mating behaviours, observed copulatory ties, and/or by calculating backwards from the birth of pups. The date on which oestrus was estimated to start using these methods was designated as day 0. Aligning data to a designated 'day 0' is commonly done in reproductive physiology studies (e.g. Carlson & Gese, 2008; Gudermuth et al., 1998; Walker et al., 2002). Because breeding is synchronized within an area (Sillero-Zubiri et al., 1998), oestrus dates were calculated for all females within an area (Web Valley or Sanetti Plateau) each year. We estimated oestrus to last for 15 days, and to allow for females coming into oestrus at slightly different times we included five days before and after the estimated 'oestrus time'. Thus, the 'oestrus time' was determined to be between days -5 and +20. Since it was

difficult from our behavioural observations to distinguish late pro-oestrus and early oestrus, our ‘oestrus time’ probably includes late pro-oestrus as well. Similarly, we aligned the time of pregnancy to a ‘pregnancy date’. Pregnancy day 0 was determined by adding 15 days to the oestrus date. As with the oestrus date, 5 days were added both before and after pregnancy, to allow for females who conceived slightly earlier or later, so that ‘pregnancy’ was defined as days -5 to +65. This gave a duration of pregnancy consistent with our observations of mating and/or birth of pups.

Although all three field seasons lasted from August to February/March, not all females were sampled for an equal period of time, especially for the period of time in oestrus days. Because the 2008 mating season was unusually late, more samples from days before oestrus day -5 are available for some females. Three females (MEG06, SOD02 in 2008, SOD04) were also sampled between March and August 2009. Finally, several females were sampled for shorter periods of time because they died/disappeared as a result of the 2008-09 rabies epizootic. Numbers of samples collected for different females also varied in function of the field observations and the likelihood of encountering a given female at regular intervals. Although an effort was made to track each pack twice a week, biweekly samples could not always be collected from every female due to circumstances such as bad weather (fog) or failure to find a specific focal female.

Observations of wolf mating behaviour and aggressive behaviour were recorded whenever they occurred. However, aggressive interactions were seldom recorded in this study. To increase our sample size and further study patterns of aggressive behaviour between dominant and subordinate females, data collected by EWCP between 1988 and 2010 was incorporated into our analysis.

To compare rates of aggression between dominants and subordinates we used χ^2 tests, with Yates corrections if expected values were less than 5 (Sokal &Rohlf, 1981). To ensure independence of datapoints observations relating to the same female were recorded only once, so that, for example, if one female was seen mating several times in the same mating season, this was treated as one observation. To compare oestradiol, progesterone and cortisol levels between dominant and subordinate females, and between times of the year (e.g. oestrus and non-oestrus), general linear models (GLMs) were used (e.g. Goymann et al., 2001; Strier et al., 1999), with blocking for individual females, to correct for individual variation between females (Grafen & Hails, 2002). Before using GLMs we tested that the assumptions were met. Where necessary, responses in these models were log transformed. To compare hormone levels between dominant and subordinate females, a between subject effect in these analyses, we used a single summary approach (Grafen and Hails, 2002), using two sample T tests to compare average levels of hormones in dominants and subordinates. The level of significance was set at $p \leq 0.05$. χ^2 tests were done in Microsoft Excel® and GLM analyses and T-tests were done using Minitab® statistical software.

Note on graphs: Dominant individuals are represented by blue (light blue, dark blue, aqua) colours and subordinate individuals are represented by red (red, orange, purple, pink) colours. Different hormones are represented by different symbols such as circles for oestradiol and squares for progesterone. Oestrus periods are represented by blue or green shading, time of pregnancy and/or birth is represented by pink shading. Statistically significant differences are denoted by an asterisk (*).

4.3 Results

4.3A Reproductive behaviours, breeding success and changes in dominance status

Eight of the 14 dominant females studied bred successfully, with between one and six pups emerging (Table 4.2). Three dominant females died during the 2008-09 rabies epizootic before the birth of the pups. Two of these three (SOD06 and DAR02) appeared visibly pregnant before disappearing, and a post-mortem of the third (KOT30) revealed she was in oestrus. Three dominant females became pregnant and gave birth but lost their litters (DAR02 and SOD06 in 2007 and NYA36 in 2009). Two females changed from being subordinate to dominant. One of these (SOD06) achieved dominant status after splitting from her original pack, Sodota, to form Addaa pack. Another female (MEG06) achieved dominant status after the Megity dominant male (MEG03) and female (MEG02) dispersed, leaving MEG06 in the original Megity territory with three males from an adjacent, non-focal pack.

Table 4.2: Breeding information for females included in this study

Pack	ID	Status	Pregnant?	# of pups emerged	Notes
2007					
Addaa	SOD06	Dominant	yes	0	Lost her litter
Darkeena	DAR02	Dominant	yes	0	Lost her litter
Sodota	SOD02	Dominant	yes	3	
2008					
Addaa	SOD06	Dominant	yes	0	Died in the 2008-09 rabies epizootic
Darkeena	DAR02	Dominant	yes	0	Died in the 2008-09 rabies epizootic
Darkeena	DAR10	Subordinate	no	n/a	Died in the 2008-09 rabies epizootic
Darkeena	DAR12	Subordinate	no	n/a	Died in the 2008-09 rabies epizootic
Darkeena	DAR14	Subordinate	no	n/a	Died in the 2008-09 rabies epizootic
Kotera	KOT30	Dominant	no	n/a	Died in the 2008-09 rabies epizootic
Megity	MEG02	Dominant	yes	1	
Megity	MEG06	Subordinate	no	n/a	
Mulamo	DAR06	Dominant	yes	5	
Mulamo	DAR08	Subordinate	no	n/a	Died in the 2008-09 rabies epizootic
Sodota	SOD02	Dominant	yes	6	
2009					
Megity	MEG06	Dominant	yes	5	
Sodota	SOD02	Dominant	yes	3	
Sodota	SOD04	Subordinate	no	n/a	
BBC	BBC32	Dominant	yes	3	
BBC	BBC42	Subordinate	no	n/a	
Dumal	DUM02	Dominant	yes	3	
Dumal	DUM04	Subordinate	no	n/a	
Nyala	NYA36	Dominant	yes	0	Lost her litter
Nyala	NYA32	Subordinate	no	n/a	

Recorded mating events included females standing tail aside ('breeding stance'), males sniffing/licking females' genitals, mounts and copulatory ties. Over the course of the three mating seasons, 31 mating events were observed (Table 4.3, see Appendix II). Thirteen mating events involved the dominant male and female of a pack, and five involved the dominant female and a subordinate male of the same pack. In three of these cases, the subordinate male was chased away from the dominant female by the pack's dominant male. Eleven mating events involved a dominant female and a male of unidentified status and pack. Only two mating events involved subordinate females. In one case, subordinate female DAR12 was seen soliciting Darkeena's dominant male, DAR03, who mounted her, but there was no copulatory tie. The dominant female, DAR02, was nearby at the time but did not interfere. Subordinate female BBC42 was seen mounted by a male from a neighbouring pack, although there was no copulatory tie. These data show that dominant females are more likely to mate than subordinate females (χ^2, p=0.035). While all the mating events involving dominant females were observed during the annual mating season (oestrus days -5 to +20), the two mating events involving subordinate occurred later than the annual mating season (oestrus day 27 for DAR12 and oestrus day 47 for BBC42).

Table 4.3: Mating behaviour observations recorded during this study

Female status	**Male status**	**# of mating events**
Dominant	Dominant	13
Dominant	Subordinate	5
Dominant	From another pack/not identified	11
Subordinate	Dominant	1
Subordinate	From another pack/not identified	1
Total		**31**

4.3B Female aggressive behaviours in inter and intra pack interactions

During this study 35 aggressive interactions between wolves that involved at least one female were observed. Between 1988 and 2010, 70 aggressive interactions involving at

least one female were recorded by EWCP. For 16 of these, the nature of the interaction (inter-pack or intra-pack) could not be determined. Of the 54 remaining aggressive interactions, 22 were inter-pack and 32 were intra-pack. The rank of females involved in inter-pack aggressions could often not be determined, so these observations were excluded. The dominance rank of females could be determined for 30 of the 32 intra-pack aggressions involving females, so we included these data (Table 4.4).

Table 4.4: Aggressive inter and intra-pack interactions involving females recorded in this study and by EWCP between 1988 and 2010

Interpack aggression	
Instigated by	**#of events recorded**
Dominant female alone	1
Dominant female and males	15
Dominant female and subordinate female	1
Subordinate female and males	2
Unidentified	10
Total	*29*
Intrapack aggression	
Description	**#of events recorded**
Dominant female behaves aggresively to subordinate female	13
Subordinate female behaves aggresively to lower ranking female	10
Dominant male behaves aggressively to a subordinate female	2
Subordinate male behaves aggressively to a subordinate female	7
Dominant female behaves aggresively to a juvenile of unknown sex	1
Dominant male steals food from dominant female	2
Dominant male and female chase subordinate male	1
Total	*36*
Total overall	*65*

The majority of our 35 observed aggressive interactions (n=29) were between packs and consisted of members of one pack chasing members of another pack or floaters (Ethiopian wolves who are not members of an existing pack), with little or no physical contact. Seventeen of the 29 inter-pack aggressions were instigated by wolves that included the dominant female and males (n=15), the dominant female and a subordinate female (n=1), or only the dominant female (n=1). In only two cases was aggression instigated by the subordinate female, together with a male. In the remaining cases aggression was instigated

by unidentified individuals or males. These data indicate that dominant females are more likely than subordinates to instigate inter-pack aggression (χ^2, p=0.035). In most cases the status of the wolves on the receiving end of the aggression could not be determined, as focal pack territories often bordered on non-focal pack territories, so we could not reliably test if dominant females are also more likely to be targeted by inter-pack aggressions.

The total recorded intra-pack aggressions included 13 cases where a dominant female acted aggressively to a subordinate female, and 10 cases where a subordinate female acted aggressively to a lower ranking female. On a further nine occasions, males, either dominant (n=2) or subordinate (n=7) acted aggressively to a subordinate female. Dominant females were never the target of intra-pack aggression. These observations show that although dominant and subordinate females are equally likely to act aggressively to lower ranking females (Yates correction χ^2, p=0.72), subordinate females are more likely to be on the receiving end of intra-pack aggressions than dominant females (Yates correction χ^2, p=0.003).

4.3C Oestrus: Mating behaviour and oestradiol levels

Dominant females had significantly higher average oestradiol levels during oestrus than at other times of the year (GLM, $F_{1,10}$=12.46, p<0.001). In contrast, subordinate females showed no significant differences in average oestradiol levels between oestrus and other times of the year (GLM, $F_{1,8}$=0.4, p=0.525). Oestradiol levels were significantly higher in dominant females than in subordinate females at the time of oestrus (single summary statistic two sample T-test, DF=16, p=0.001), but not at other times (single summary statistic two sample T-test, DF=12, p=0.38, Fig. 4.3).

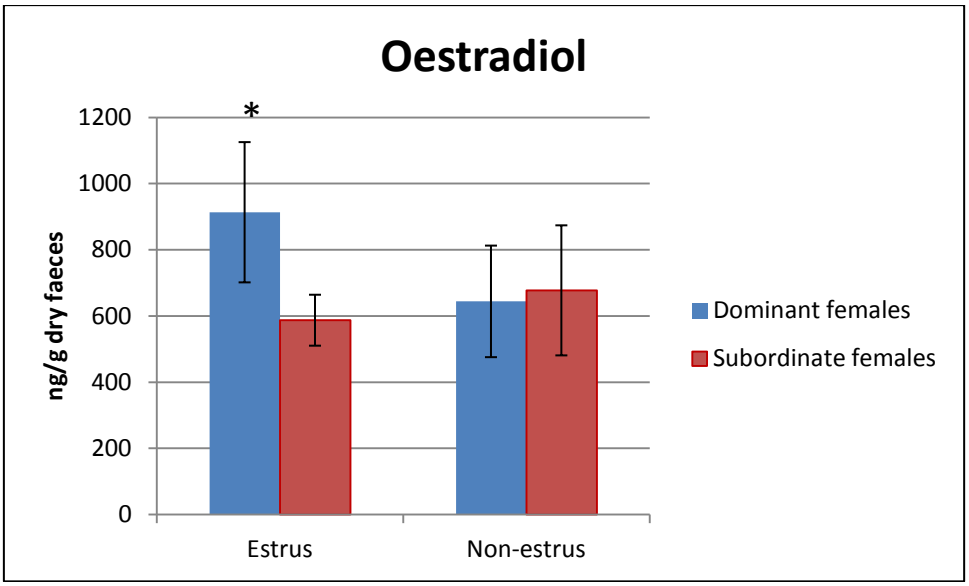

Figure 4.3: Oestradiol levels of dominant (n=11) and subordinate (n=9) females in oestrous (days -5 to +20) and not in oestrus (all other days). The asterisk (*) denotes a significant difference. Note: the bars represent the combined data from all dried samples. Error bars denote standard error of individual wolves.

All dominant females were judged to be in oestrus either by being seen mating and tied (n=5), showing mating behaviour such as standing tail aside, or being mounted without a tie (n=1), by becoming visibly pregnant (n=2) or oestrus was confirmed by the birth of pups approximately two months later (n=5). In one case, a female (KOT30) was found dead during the 2008-09 rabies epizootic and was confirmed to be in oestrus through a post mortem examination. Thirteen of the fourteen dominant females showed an oestradiol peak (an increase of at least 2.6 fold over baseline values) during the annual mating season. In contrast, none of the nine subordinate females showed an oestradiol peak during the annual mating season (Table 4.5).

Table 4.5: Oestradiol increases in females during oestrus (days -5 to +20). Bold type indicates dominant females who showed a clear oestradiol peak (>2.6 fold increase over baseline levels). Underlined type indicates dominant females who did not show a clear oestradiol peak despite being in oestrous. Italic type indicates subordinate females

Year	Pack	Female	Baseline E2	Highest E2 during oestrus	Increase	Oestrus day	Status
2007	**Darkeena**	**DAR02**	**182.7**	**503.0**	**2.8**	**21**	**Dominant**
2007	**Sodota**	**SOD02**	**241.0**	**623.3**	**2.6**	**15**	**Dominant**
2007	**Addaa**	**SOD06**	**193.3**	**778.9**	**4.0**	**13**	**Dominant**
2008	**Darkeena**	**DAR02**	**401.5**	**2586.8**	**6.4**	**15**	**Dominant**
2008	*Darkeena*	*DAR10*	*659.1*	*871.2*	*1.3*	*-2*	*Subordinate*
2008	*Darkeena*	*DAR12*	*564.7*	*709.4*	*1.3*	*5*	*Subordinate*
2008	*Darkeena*	*DAR14*	*510.1*	*784.0*	*1.5*	*19*	*Subordinate*
2008	**Kotera**	**KOT30**	**472.2**	**1417.0**	**3.0**	**8**	**Dominant**
2008	**Megity**	**MEG02**	**551.0**	**5183.0**	**9.4**	**20**	**Dominant**
2008	*Megity*	*MEG06*	*514.2*	*737.9*	*1.4*	*7*	*Subordinate*
2008	**Mulamo**	**DAR06**	**408.6**	**1972.6**	**4.8**	**19**	**Dominant**
2008	*Mulamo*	*DAR08*	*439.9*	*439.8*	**1.0**	*19*	*Subordinate*
2008	**Addaa**	**SOD06**	**526.3**	**1953.5**	**3.7**	**20**	**Dominant**
2008	**Sodota**	**SOD02**	**557.8**	**2110.6**	**3.8**	**16**	**Dominant**
2009	Sodota	SOD02	598.0	1476.4	2.5	20	Dominant
2009	*Sodota*	*SOD04*	*575.7*	*717.0*	*1.2*	*15*	*Subordinate*
2009	**Megity**	**MEG06**	**569.6**	**3648.1**	**6.4**	**-4**	**Dominant**
2009	**BBC**	**BBC32**	**411.3**	**1091.0**	**2.7**	**21**	**Dominant**
2009	*BBC*	*BBC42*	*431.3*	*503.5*	*1.2*	*16*	*Subordinate*
2009	**Dumal**	**DUM02**	**553.0**	**1953.2**	**3.5**	**18**	**Dominant**
2009	*Dumal*	*DUM04*	*552.3*	*1091.2*	*2.0*	*18*	*Subordinate*
2009	**Nyala**	**NYA36**	**495.0**	**2067.6**	**4.2**	**20**	**Dominant**
2009	*Nyala*	*NYA32*	*465.1*	*1093.5*	2.4	*-1*	*Subordinate*

Dominant females had significantly higher oestradiol levels (as a proportion of baseline values) than subordinate females during the annual mating season (oestrus), whereas subordinate females' oestradiol levels remained relatively constant between oestrus weeks -11 and +11 (Fig. 4.4).

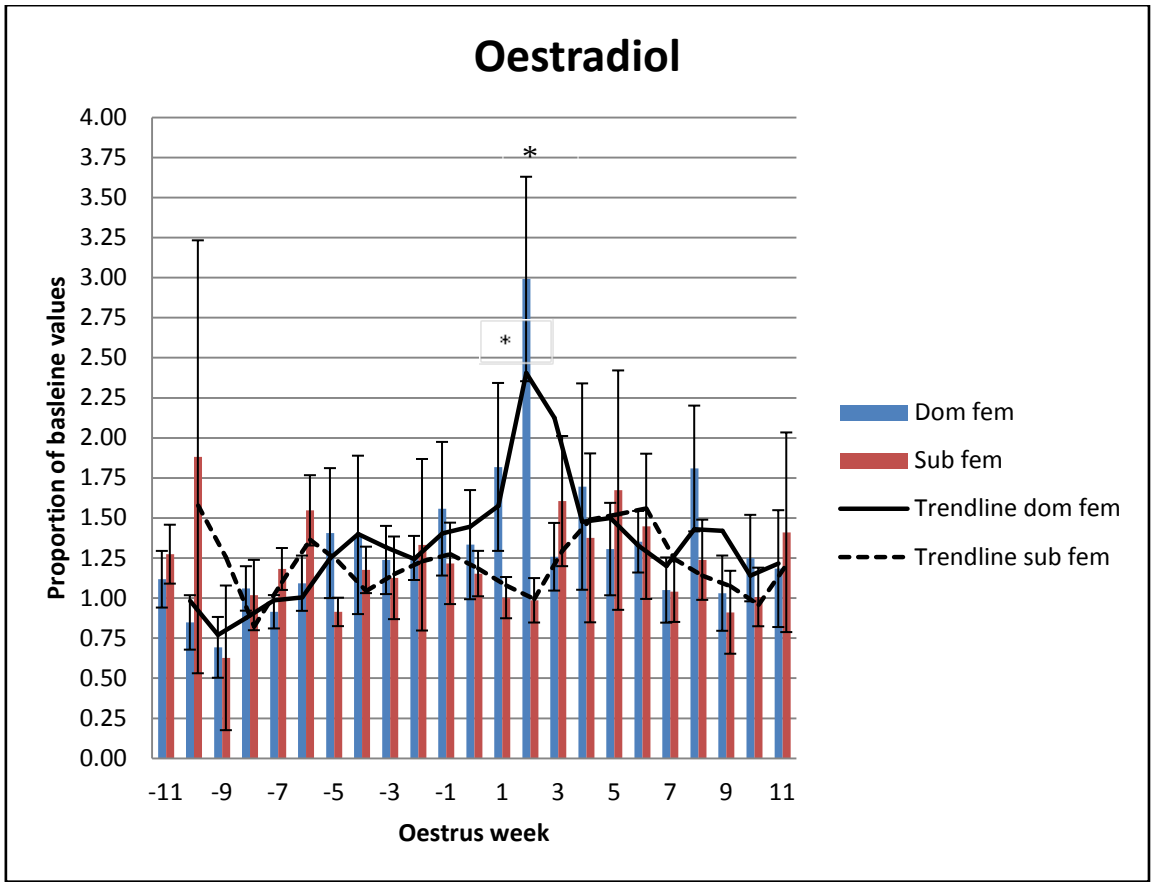

Figure 4.4: Average weekly oestradiol levels of dominant (n=14) and subordinate (n=9) females expressed as a proportion of baseline values. Trendlines are calculated as averages of two consecutive points. The asterisks (*) denote a significant difference. Error bars denote standard error of individual wolves.

Thirteen of the 14 dominant females showed an oestradiol peak around the time they started showing oestrus behaviour such as mating (Figs. 4.5 and 4.6A). Female SOD06 was originally a subordinate female in Sodota pack. She showed an oestradiol peak at the same time as her dominant female (SOD02, Fig. 4.5A) although we did not observe any oestrus behaviour from SOD06 at the time. SOD06 then split from Sodota pack, and came into oestrus again a month later. She was observed mating and became pregnant, although no pups emerged. Dominant females MEG02, SOD02 and DAR02 in 2008 (Fig. 4.5B), DAR06, KOT30 and SOD06 in 2008 (Fig. 4.5C), and females MEG06 in 2009, DUM02 and NYA36 (Fig. 4.5D) all showed clear oestradiol peaks between oestrus days -5 and +20.

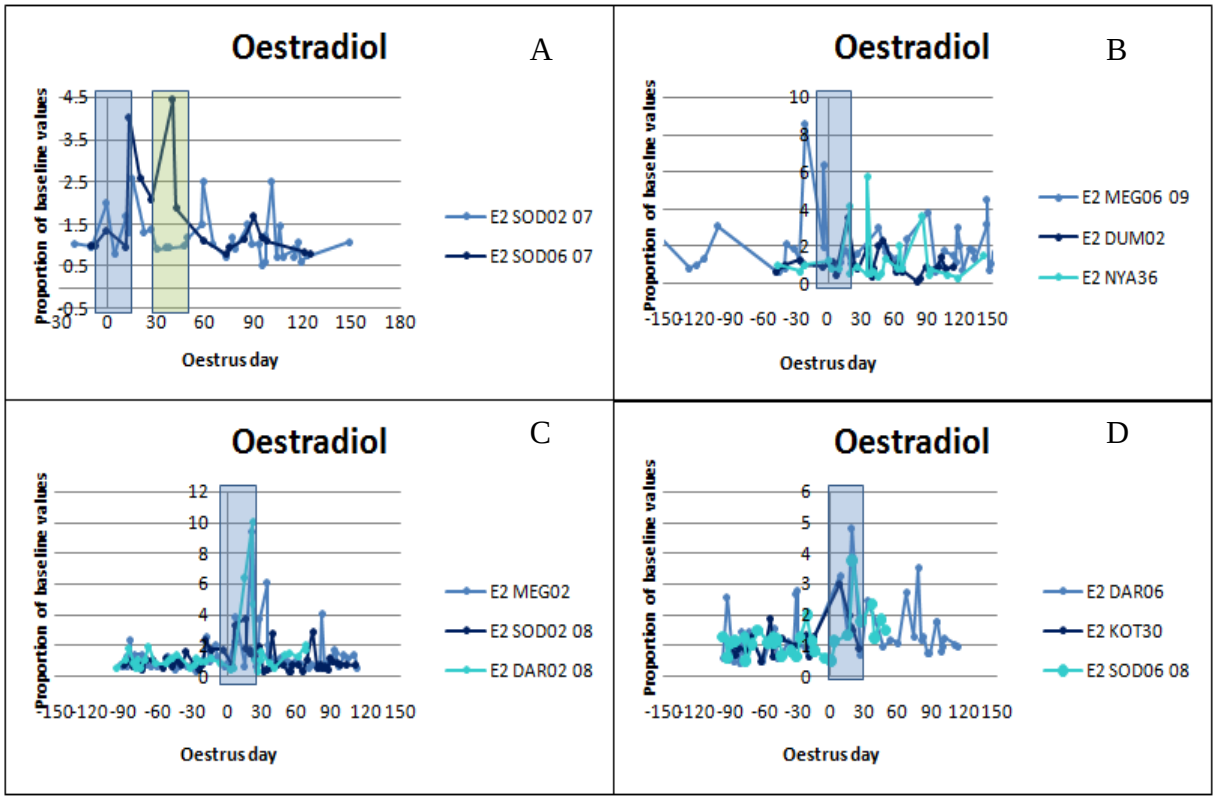

Figure 4.5: Oestradiol (E2) increases in dominant females SOD02 and SOD06 in 2007 (A), MEG06 in 2009, DUM02, NYA36, (B) MEG02, SOD02 and DAR02 in 2008 (C), DAR06, KOT30 and SOD06 in 2008 (D). Blue shading shows the time of oestrus (days -5 to +20). Green shading shows the time SOD06 in 2007 came into oestrus (days +27-+42). Note that DAR02 in 2008, SOD06 in 2008, and KOT30 died in the 2009-09 rabies epizootic.

Two dominant females (BBC32 and DAR02 in 2007) both showed an oestradiol peak on oestrus day 21 (Fig. 4.6A). Both females became pregnant and gave birth, although DAR02 lost her litter. For both of these females the estimated dates of pregnancy indicate that their coming into oestrus on day +21 is plausible, so it may be that they came into oestrus slightly later than the other females. Female SOD02 in 2009 shows an unusual pattern (Fig. 4.6B). SOD02 has large increases in oestradiol on oestrus days -29 and +60, but no increase between oestrus days -5 and +20. There is a 2.46 increase on oestrus day 21, but by our observations of pregnancy and birth, SOD02 was already pregnant by this time. This female therefore has an unusual profile in that we did not manage to detect an oestradiol peak associated with oestrus.

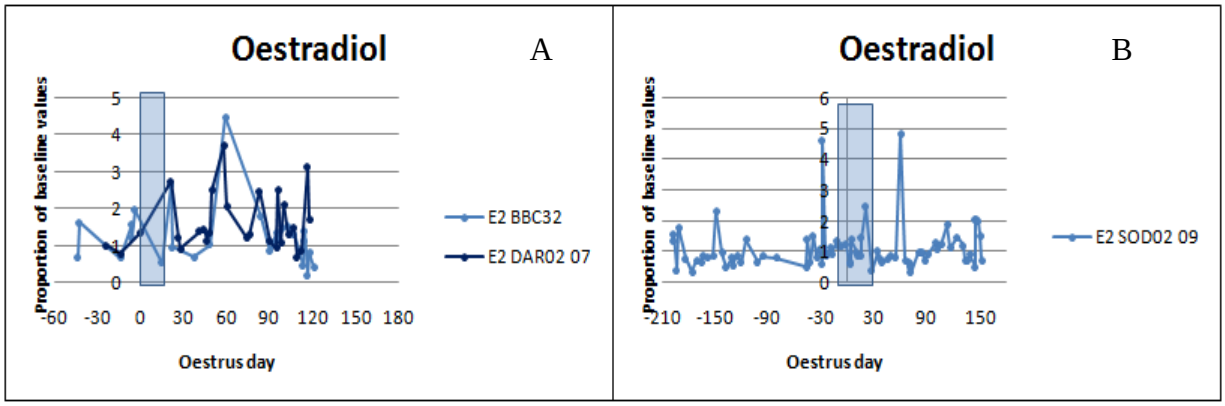

Figure 4.6: Oestradiol increases in dominant females BBC32 and DAR02 in 2007 (A) and SOD02 in 2009 (B). Blue shading shows the time of oestrus (days -5 to +20).

None of the nine subordinate females (excluding SOD06, who later became dominant) showed an oestradiol peak between days -5 and 20 (Table 4.5). The nine subordinate females can be divided into three sub-groups (Figure 4.7). The first group includes females whose oestradiol levels did not increase >2.4 fold of baseline values at any point between oestrus days -150 and +145 (females SOD04, DAR08, NYA32 and MEG06 in 2008, Fig. 4.7A). The second group consists of females DAR12 and BBC42 who came into oestrus outside of the annual mating season. DAR12 showed an oestradiol increase of 4.2 fold on day 29 (Fig. 4.7B), and was confirmed to be in oestrus when she was observed standing tail aside for the pack's dominant male, who mounted her, although there was no tie. Female BBC42 showed a 6.7 fold increase in oestradiol on day 43 (Fig. 4.7B). BBC42 was mounted by a male around this time, although there was no copulatory tie. The third group consists of females who show oestradiol peaks outside of the annual mating season, but were not seen showing any oestrus behaviour. This group includes DAR10, DAR14 and DUM04 (Fig. 4.7C). DAR10 and DAR14 showed oestradiol peaks on days -6 and +29 respectively, but were not seen showing any oestrous behaviour (Fig. 4.8C). DUM04 showed oestradiol increases on days 46, 86, 112, and 130, but did not show any oestrous behaviour (Fig. 4.7C).

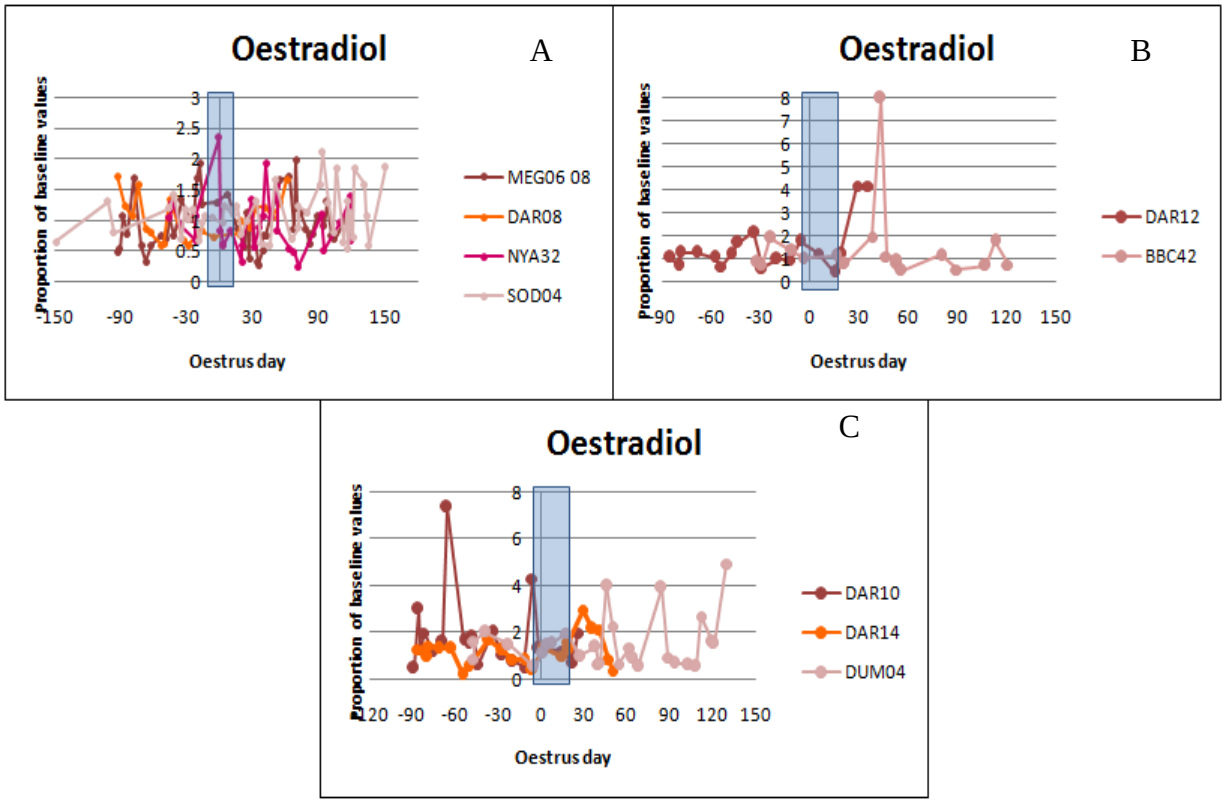

Figure 4.7: Oestradiol increases in subordinate females MEG06 in 2008, DAR08, NYA32 and SOD04 (A), DAR12 and BBC42 (B) and DAR10, DAR14 and DUM04 (C). Blue shading shows the time of oestrus (days -5 to +20). Note that DAR08, DAR10, DAR12, and DAR14 disappeared in the 2008-09 rabies epizootic.

Between June and September 2009, female MEG06 changed status from subordinate to dominant. MEG06's pack, Megity, was greatly affected by the 2008-09 rabies epizootic, with only three of the original 23 wolves surviving. The pack's dominant male (MEG03) and female (MEG02) survived and MEG02 gave birth and one pup emerged in January 2009. Between June and September 2009, however, MEG02 and MEG03 dispersed from Megity territory. MEG06 then lured three males from a neighbouring non-focal pack (Alando pack), into Megity territory and became the dominant (and only) female in the pack. She mated, became pregnant and five pups emerged in November 2009. When MEG06 was subordinate, her oestradiol levels remained low throughout the breeding season, whereas MEG02 showed a large oestradiol peak during the annual mating season (Fig. 4.8). After attaining dominant status, however, MEG06 showed a large oestradiol peak during the annual mating season (Fig. 4.8), mated and bred successfully.

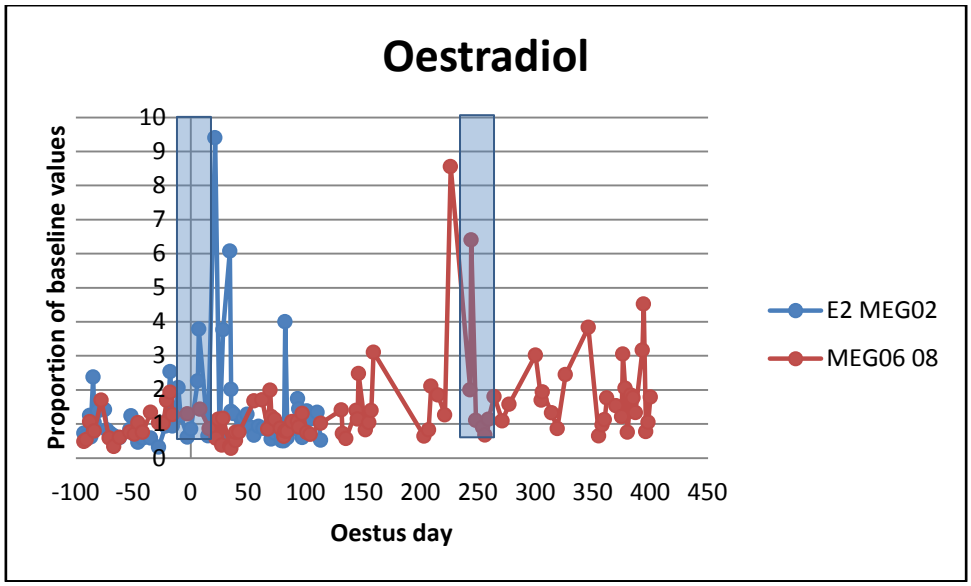

Figure 4.8: Oestradiol levels of MEG02 and MEG06 in 2008 (when she was subordinate to MEG02) and MEG06 in 2009, when she was the dominant (and only) female in the pack. Shading shows the time of estrous (days -5 to +20)

4.3C Pregnancy and pseudopregnancy: progesterone levels in dominant and subordinate females

Both dominant (GLM, $F_{1,9}$=30.45, p<0.001) and subordinate (GLM, $F_{1,8}$=9.73, p=0.002) females had significantly higher progesterone levels between pregnancy days -5 to +65 than at other times of the year (Fig. 4). Progesterone levels did not differ between dominant and subordinate females between days -5 to +65 (single summary statistic two sample T-test, DF=16, p=0.751) nor on all other days (single summary statistic two sample T-test, DF=15, p=0.084).

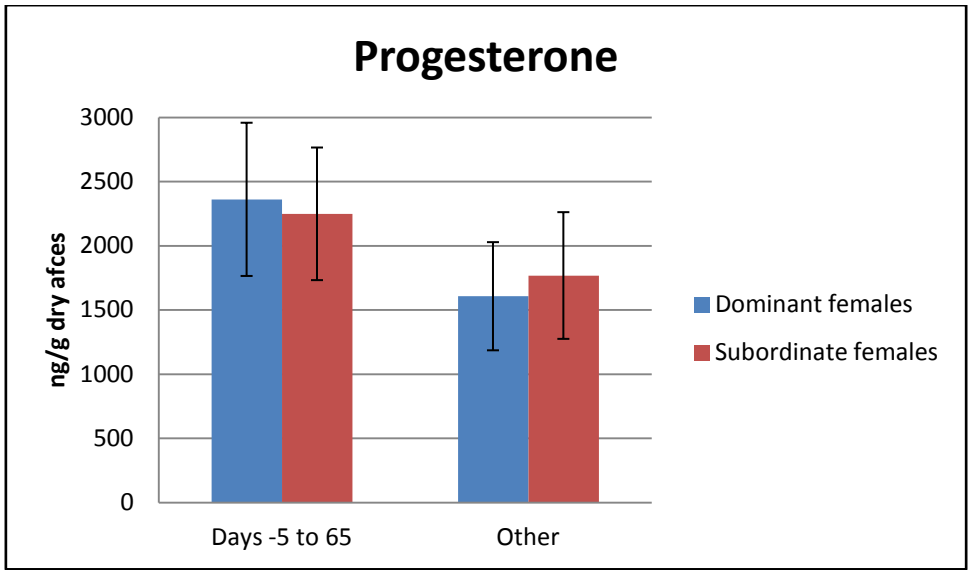

Figure 4.9: Progesterone levels of dominant (n=11) and subordinate (n=9) females between pregnancy days -5 to +65 and all other days of the field season. Note: the bars represent the combined data from all dried samples. Error bars denote standard error of individual wolves.

Progesterone levels (as a proportion of baseline values) of dominant and subordinate females were usually not significantly different, with significantly higher levels in dominants only in oestrus weeks 2 and 6 (Fig. 4.10).

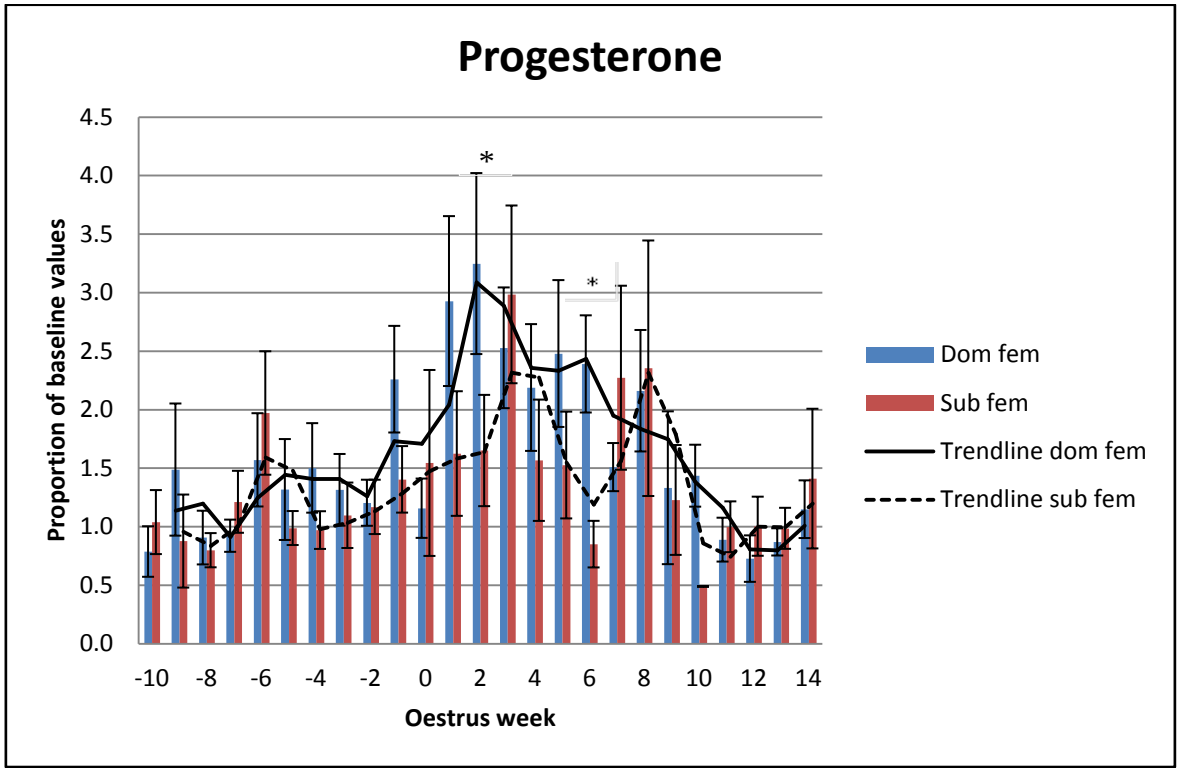

Figure 4.10: Average weekly progesterone levels of dominant (n=14) and subordinate (n=9) females expressed as a proportion of baseline values. Trendlines are calculated as averages of two consecutive points. The asterisks (*) denote a significant difference Error bars denote standard error of individual wolves.

Ten of the thirteen pregnant females showed an increase in progesterone whilst they were pregnant (Fig. 4.11). Progesterone levels generally showed a sharp increase in early pregnancy, with levels decreasing later in pregnancy.

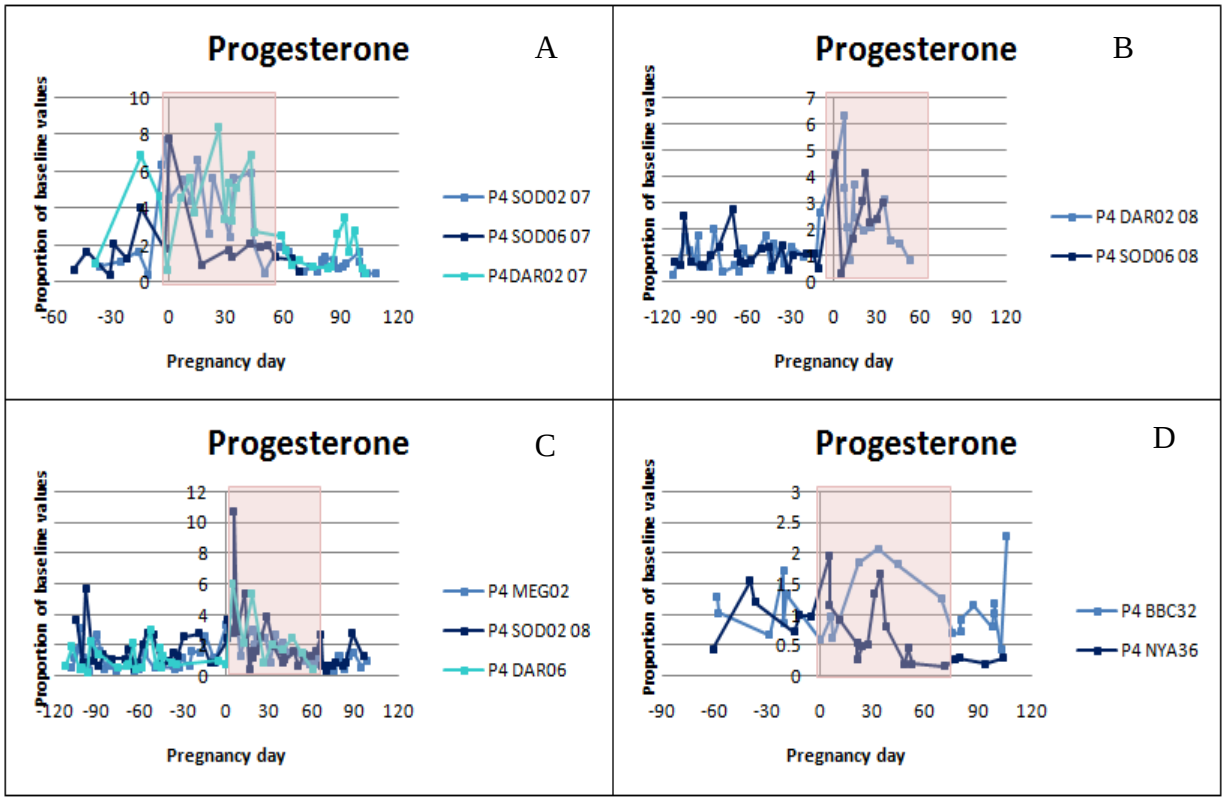

Figure 4.11: Progesterone (P4) levels of dominant females SOD02, SOD06 and DAR02 in 2007 (A), DAR02 and SOD06 in 2008 (B), MEG02, SOD02 in 2008 and DAR06 (C) and BBC32 and NYA36 (D). Shading shows the time of pregnancy (days -5 to +65). Note that SOD06 and DAR02 disappeared in the 2008-2009 rabies epizootic.

Three dominant females, including DUM02 (Fig. 4.12A) and MEG06 and SOD02 in 2009 (Fig. 4.12B) did not show clear increases in progesterone during pregnancy. All three females were confirmed to be pregnant between days – 5 and +65 by the birth and emergence of pups. Female DUM02 showed high levels of progesterone starting on day -38, but fairly low levels between days -5 and +65. Female MEG06 in 2009 showed a sharp increase in progesterone starting on day -36. Similarly, female SOD02 showed high levels of progesterone starting on day -50. For all three females these increases in progesterone occurred well before they became pregnant.

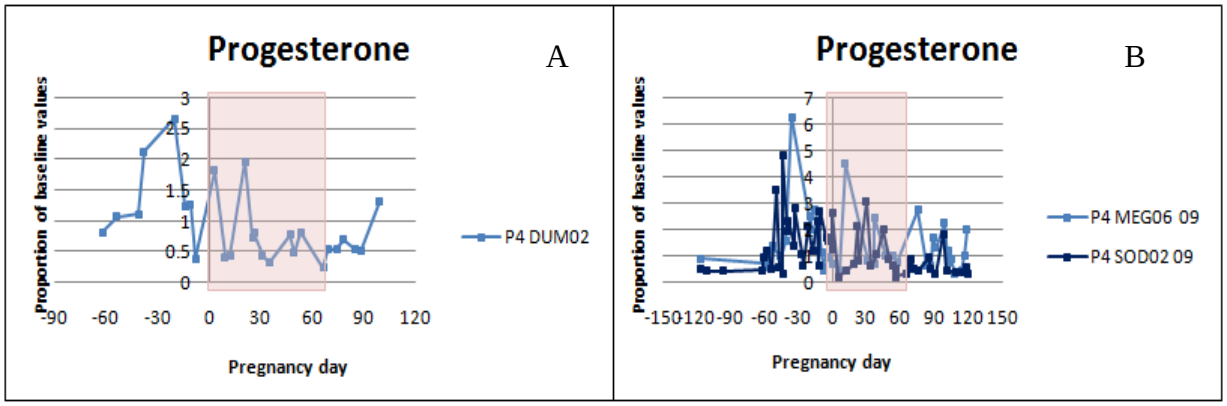

Figure 4.12: Progesterone (P4) levels of dominant females DUM02 (A) and MEG06 and SOD02 in 2009 (B). Shading shows the time of pregnancy (days -5 to +65).

Seven of the nine subordinate females in this study also showed increased levels of progesterone between pregnancy days -5 and +65 (Fig. 4.14A, B, C). Four of these females, DAR08, DAR10, DAR12, and DAR14 disappeared in the rabies epizootic before pregnancy day +65, so we cannot determine if any were pregnant or would have allosuckled the pups. MEG06 in 2008 showed physical signs of pseudopregnancy (extended abdomen, visible nipples) after oestrus day 50 (Figure 4.13), but showed no signs of having given birth, and did not allosuckle MEG02's pup. SOD04 and DUM04 both allosuckled their dominant female's pups, and both showed increased progesterone levels whilst their dominant female was pregnant (Fig. 4.14C). However, the progesterone increases in both females started approximately a month before their dominant female conceived (Fig. 4.14C). Subordinate (and non-pregnant) females NYA32 and BBC42 (Fig. 4.14D) did not show higher progesterone between days -5 and +65. Female NYA32 showed only one progesterone peak on day -15. She did not appear (pseudo)pregnant and as NYA36 lost her litter, did not allosuckle the pups. BBC42 shows an increase in progesterone starting on pregnancy day -26 and another peak on day 41. BBC42 did not appear (pseudo)pregnant nor allosuckled the pups. As BBC42 appears to have come into oestrus on pregnancy day 28 (Fig. 4.7B), the increase in progesterone on day 41 may have been related to an earlier oestradiol peak.

Figure 4.13: MEG06 showing signs of pseudopregnancy in January 2009

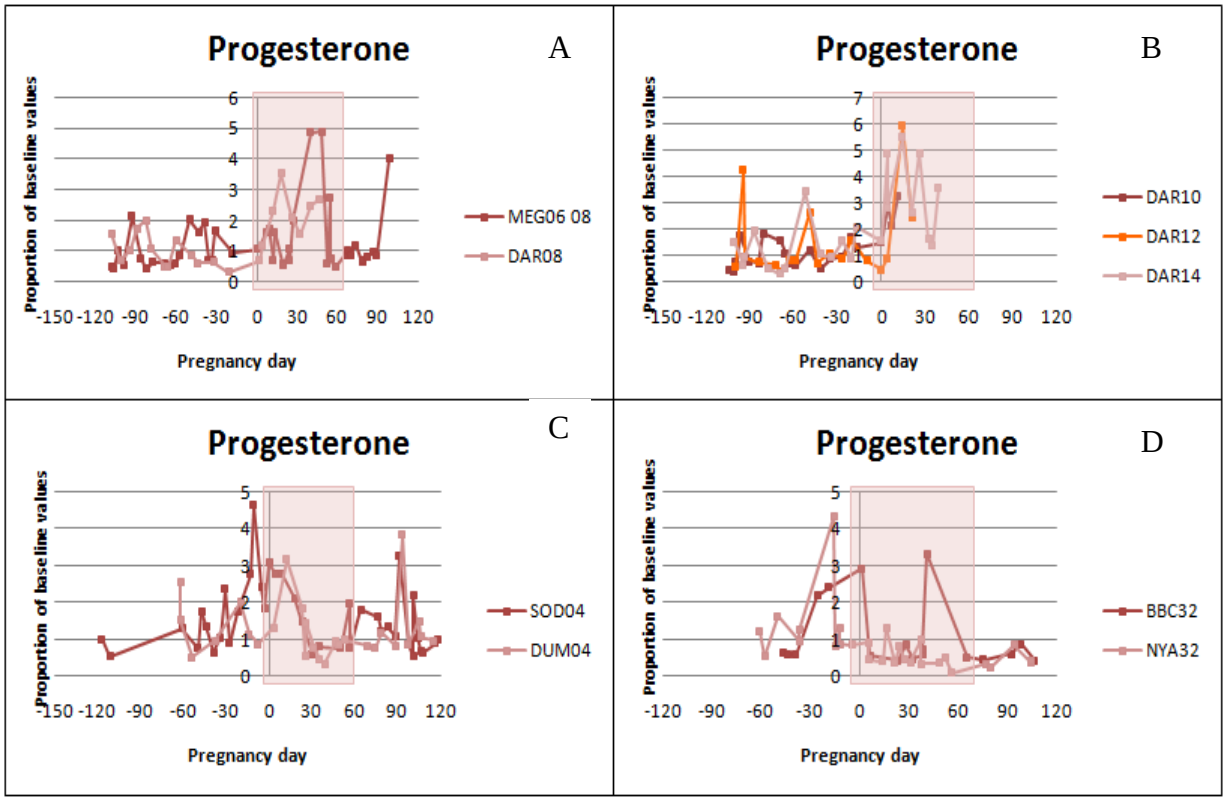

Figure 4.14: Progesterone (P4) levels of subordinate females MEG06 in 2008 and DAR08 (A), DAR10, DAR12 and DAR14 (B), SOD04 and DUM04 (C) and BBC32 and NYA32 (D). Shading shows the time of pregnancy (days -5 to +65). Note that females DAR08, DAR10, DAR12 and DAR14 disappeared in the 2008-09 rabies epidemic before their dominant females gave birth. Female MEG06 in 2008 appeared visibly pseudopregnant (Photo 1) and females SOD04 and DUM04 allosuckled their dominant female's pups.

Pseudopregnancy in subordinate females

The evidence for pseudopregnancy in subordinate females warrants a more detailed assessment. Of the nine subordinate females, seven showed evidence of pseudopregnancy,

including increased progesterone levels (SOD04, MEG06 in 2008, DAR08, DAR10, DAR12, DAR14, DUM04) and/or external signs such as an extended abdomen (MEG06 in 2008) and/or allosuckling of the pups (SOD04, DUM04).

Of the seven pseudopregnant females, two may have come into oestrus outside of the annual mating season (DAR10, DAR14), and one (DAR12) was confirmed to be in oestrus by behavioural observations. Females DAR10 and DAR14 show oestradiol peaks on oestrus days -6 and +29 respectively, although neither was seen showing any oestrus behaviour such as mating (Fig. 4.15A, B). Female DAR12 was seen soliciting the pack's dominant male around day +29, when she showed a more than fourfold increase in oestradiol levels, indicating she came into oestrus (Fig. 4.15C). All three females (DAR10, DAR12, DAR14) started showing increased levels of progesterone after the estimated conception day of their dominant females DAR02. Unfortunately, all three females disappeared in the 2008-09 rabies epizootic, so we cannot establish if they were pregnant or pseudopregnant, or if they would have allosuckled the pups.

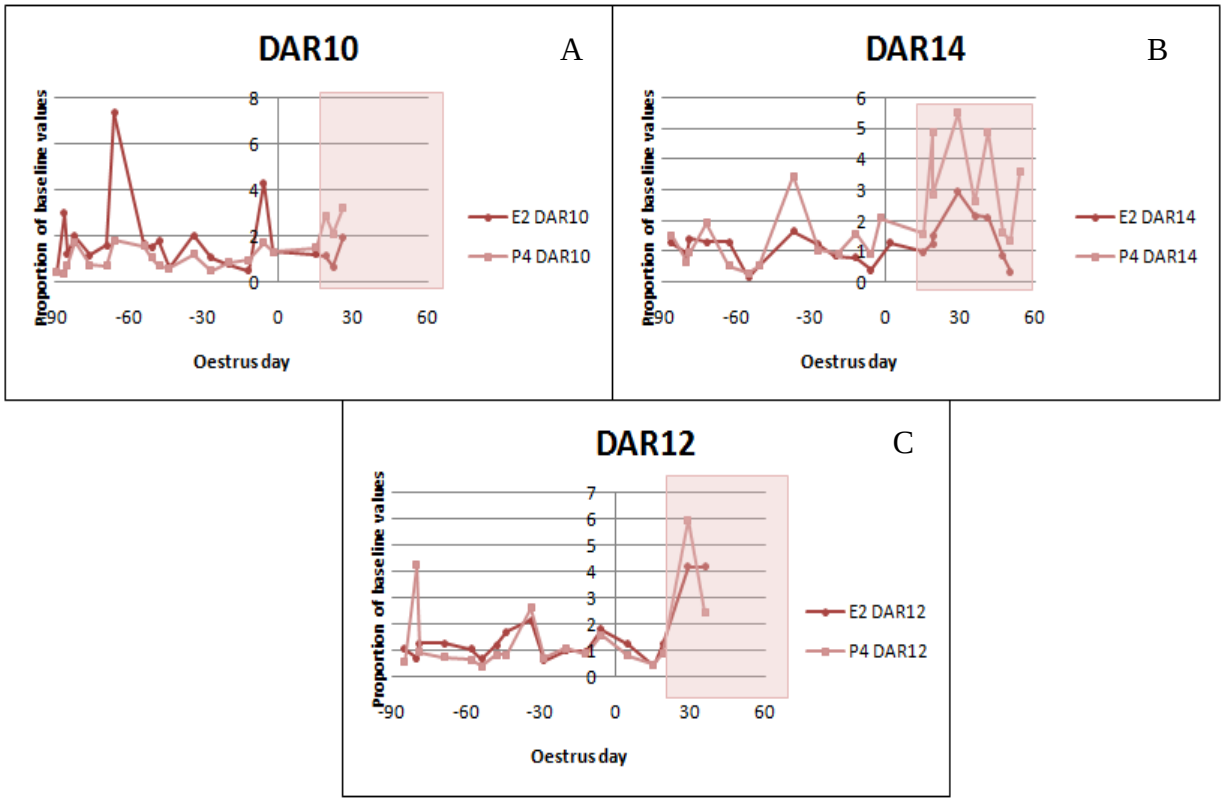

Figure 4.15: Oestradiol (E2) and progesterone (P4) levels of subordinate females DAR10 (A), DAR14 (B), and DAR12 (C). Note that all three females disappeared in the 2008-09 rabies epizootic.

The four other subordinate females who became pseudopregnant, showed no signs of oestrus either through oestradiol levels or oestrus behaviour between oestrus days -157 and +45. Females MEG06 in 2008, DAR08, and SOD04 appeared to be acyclic (Fig. 4.7A), with oestradiol levels never rising above two times baseline values. However, all became pseudopregnant, as evidenced by increased progesterone levels of 3.5-5 times baseline values, and/or physical signs such as extended abdomen and/or allosuckling the pups (Figure 4.16). Female DUM04, although showing oestradiol peaks on oestrus days 46, 84 and 130, showed no peaks between oestrus days -47 and +47, despite becoming pseudopregnant and allosuckling DUM02's pups (Fig. 4.16D).

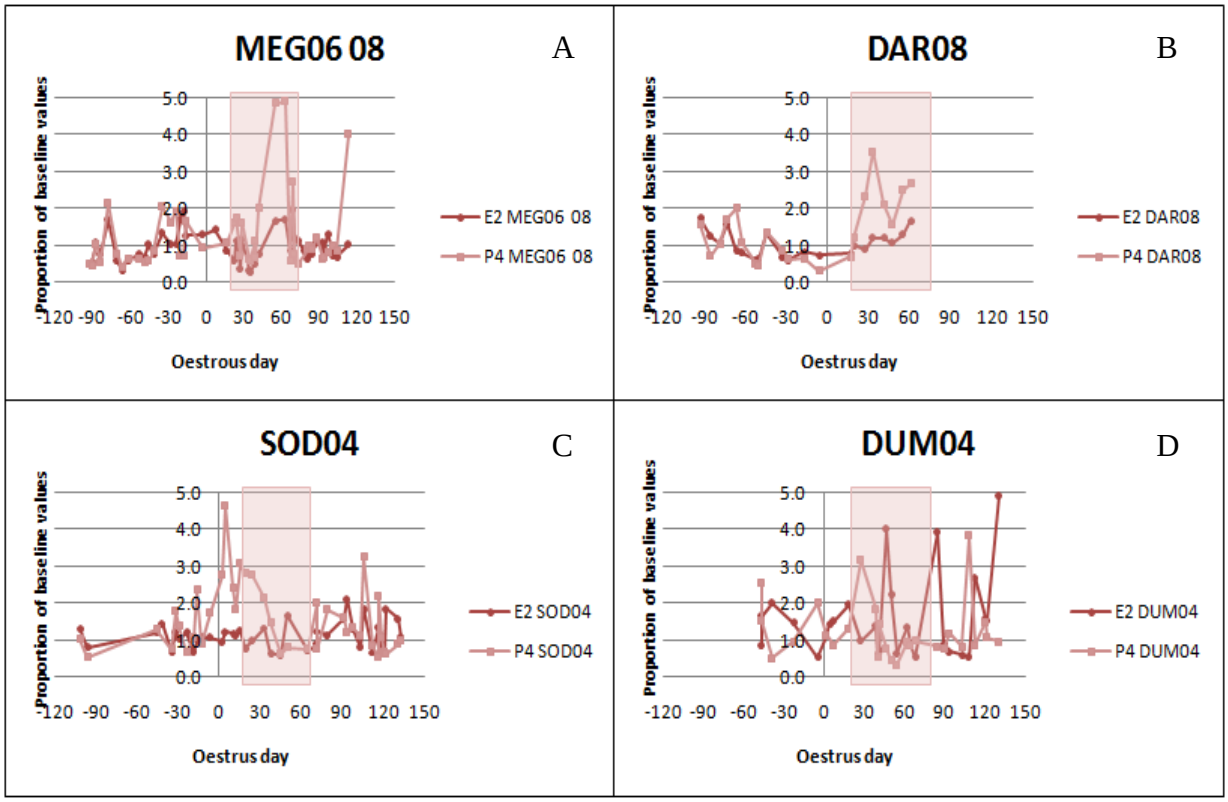

Figure 4.16: Oestradiol (E2) and progesterone (P4) levels of subordinate females MEG06 in 2008 (A), DAR08 (B), SOD04 (C) and DUM04 (D). Note that MEG06 in 2008 appeared visibly pregnant (Photo 1) and SOD04 and DUM04 allosuckled their dominant female's pups. DAR08 disappeared in the 2008-08 rabies epizootic before her dominant female gave birth.

4.3D Dominance rank and cortisol

A subset of 250 samples from female Ethiopian wolves was assayed for cortisol. Samples were selected to give a selection of dominant females (DAR02, MEG02 and MEG06 in 2009), as well as subordinate females (DAR10, DAR12, DAR14 and MEG06 in 2008). Three of these females (DAR02, MEG02 and MEG06 in 2009) became pregnant and two (MEG02 and MEG06 in 2009) gave birth. MEG06 changed from subordinate to dominant status between 2008 and 2009, and samples from MEG06 from both years were assayed for cortisol.

To compare cortisol levels between dominant and subordinate females all the results were combined. Average cortisol levels in subordinate females were higher than in dominant females, although this effect was not significant (single summary statistic two sample T

test, DF=4, p=0.199, Fig. 4.17). Cortisol levels of dominant and subordinate females did not differ significantly during oestrus (single summary statistic two sample T test, DF=4, p=0.994) nor on other days (single summary statistic two sample T test, DF=4, p=0.197, Fig. 4.18) Cortisol levels did not differ between oestrus and non oestrus for either dominant (GLM, $F_{1,2}$=1.97, p=0.162) or subordinate females (GLM, $F_{1,3}$=0.50, p=0.481, Fig. 4.18).

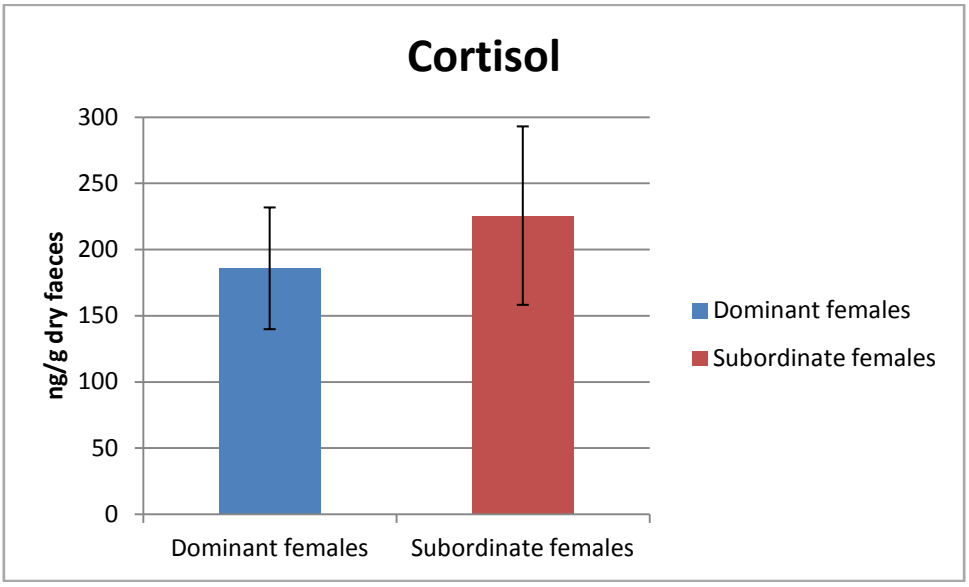

Figure 4.17: Average cortisol levels in dominant (n=3) and subordinate (n=4) females over the whole field season. Error bars denote standard error of individual wolves.

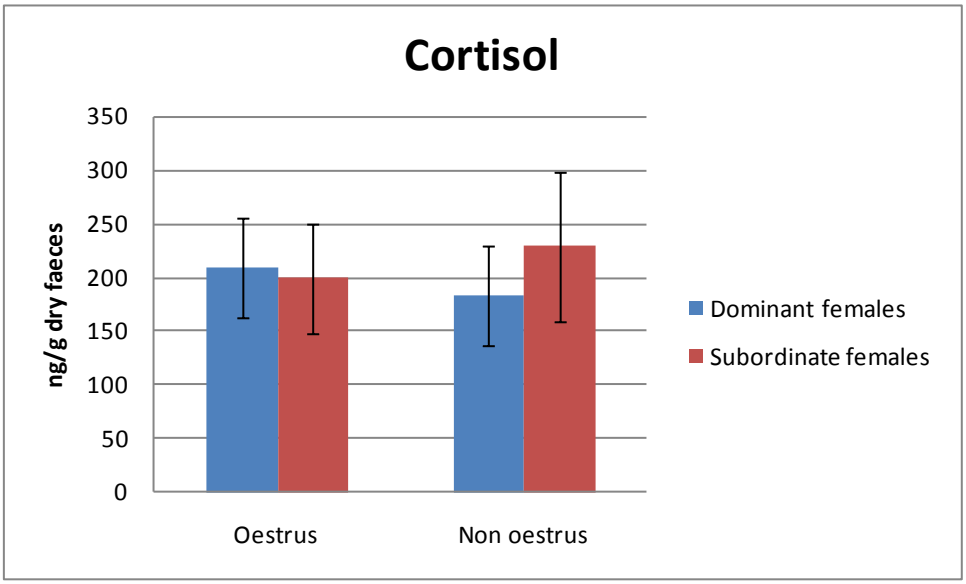

Figure 4.18: Cortisol in dominant (n=3) and subordinate (n=4) females during oestrous and non estrous. Error bars denote standard error of individual wolves.

To compare different females, cortisol values were averaged per week for all dominant and subordinate females (Fig. 4.19). Although subordinate females seemed to have higher average cortisol levels between oestrus weeks -7 and -3, we could not detect clear seasonal patterns in cortisol for individual females (Fig. 4.20, 4.21).

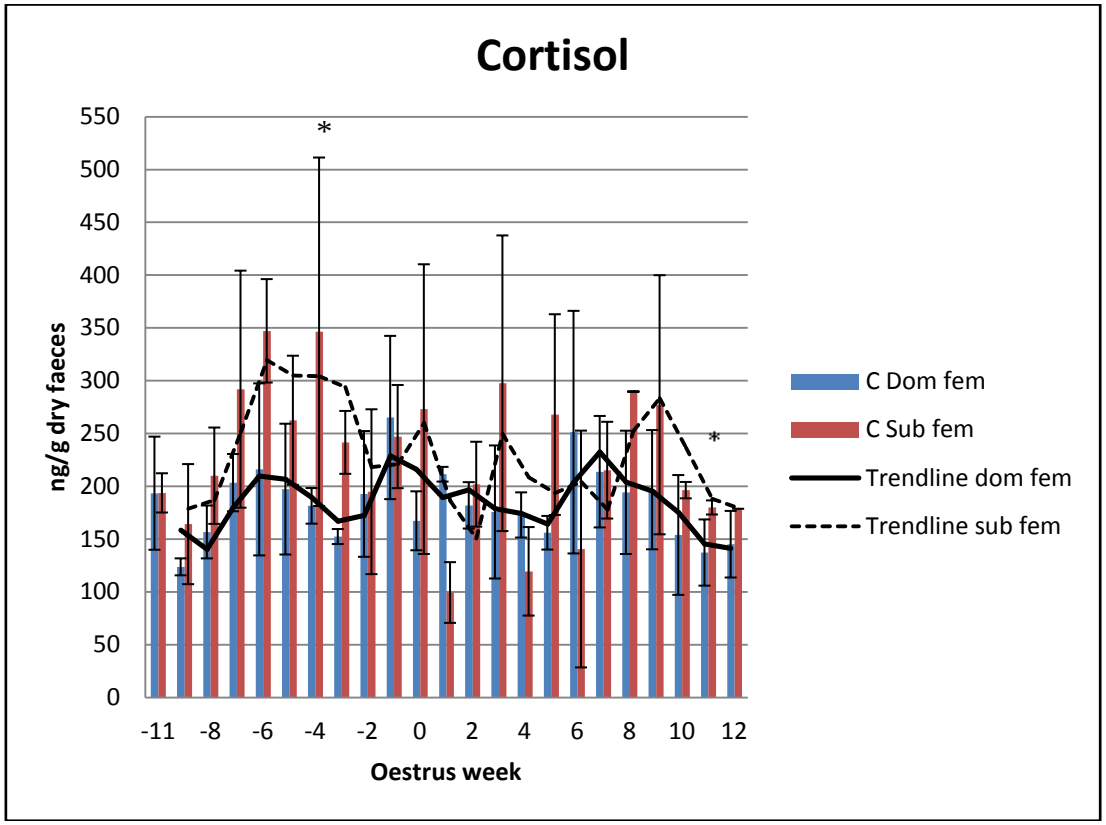

Figure 4.19: Average weekly cortisol levels of dominant (n=3) and subordinate (n=2). Trendlines are calculated as averages of two consecutive points. Asterisks (*) denote significant differences. Error bars denote standard error of individual wolves.

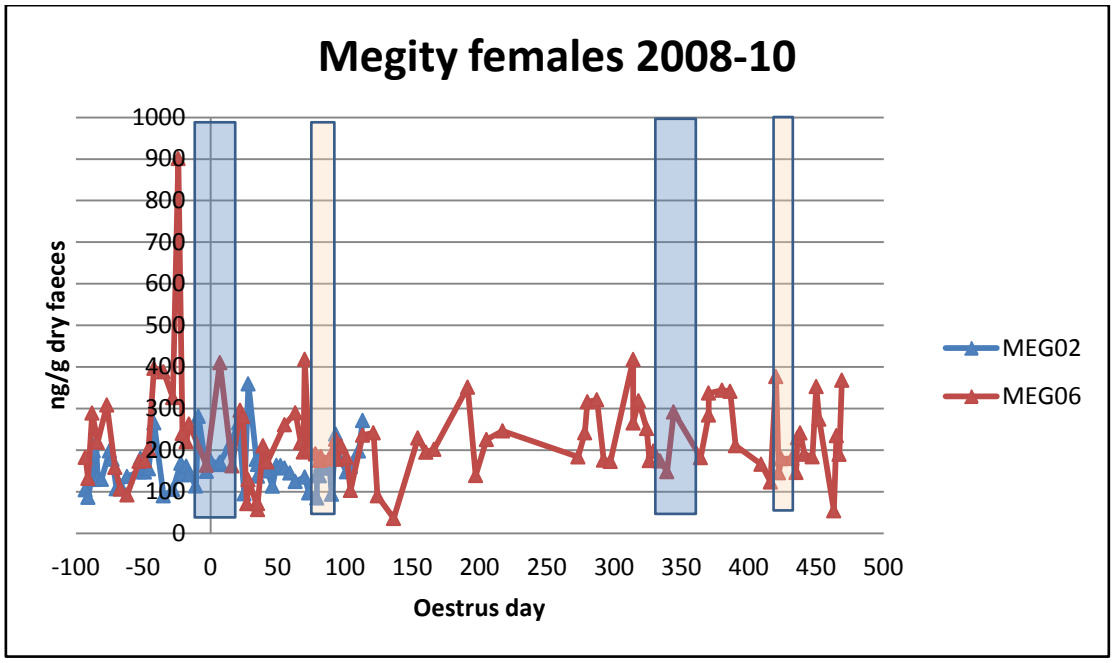

Figure 4.20: Cortisol levels of MEG02 and MEG06 between August 2008 and February 2010. Blue shading shows the time of estrous and pink shading shows the time of birth of the pups.

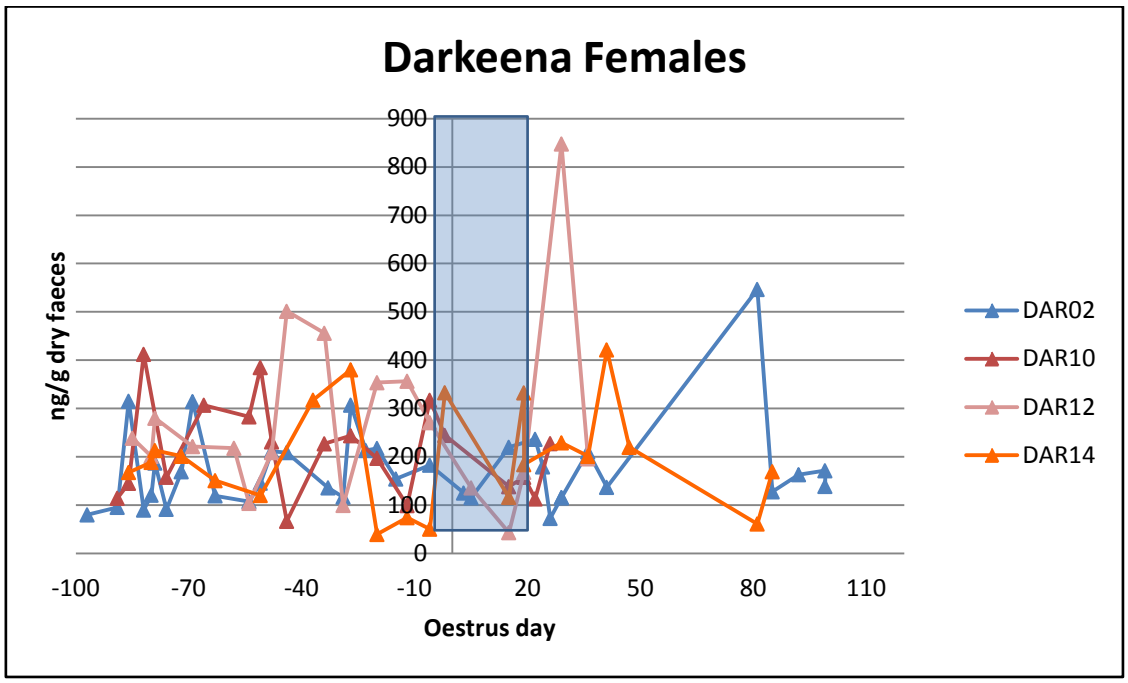

Figure 4.21: Cortisol levels of dominant female DAR02, and subordinate females DAR10, DAR12 and DAR14 in 2008.. Blue shading shows the time of oestrous. Note that all four females disappeared in the 2008-2009 rabies epidemic before the time of birth.

We analyzed samples from breeding females MEG02 and MEG06 in 2009 for cortisol. Cortisol levels in both females around the estimated day of parturition are shown in Figure 4.22. We did not detect a clear increase in cortisol before females gave birth.

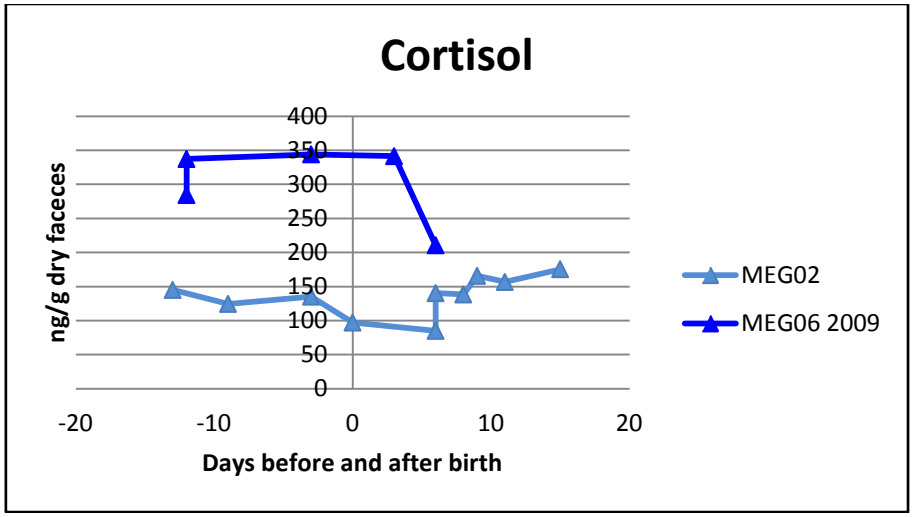

Figure 4.22: Cortisol before and after the estimated date of parturition in dominant, pregnant females (n=2)

4.3E Cortisol and Oestradiol

We compared temporal patterns in oestradiol and cortisol for seven females (Figs. 4.23 and 4.24). Although when all the data are combined, oestradiol and cortisol are not significantly correlated (Pearson correlation, p=0.06), individual females do show correlations between oestradiol and cortisol. Oestradiol and cortisol are significantly correlated in subordinate females DAR10 (Pearson correlation, p=0.031), DAR12 (Pearson correlation, p=0.005), DAR14 (Pearson correlation, p=0.003) and MEG06 in 2008 (Pearson correlation, p=0.031). Oestradiol and cortisol are not correlated in dominant females DAR02 (Pearson correlation, p=0.188), MEG02 (Pearson correlation, p=0.108) or MEG06 in 2009 (Pearson correlation, p=0.775).

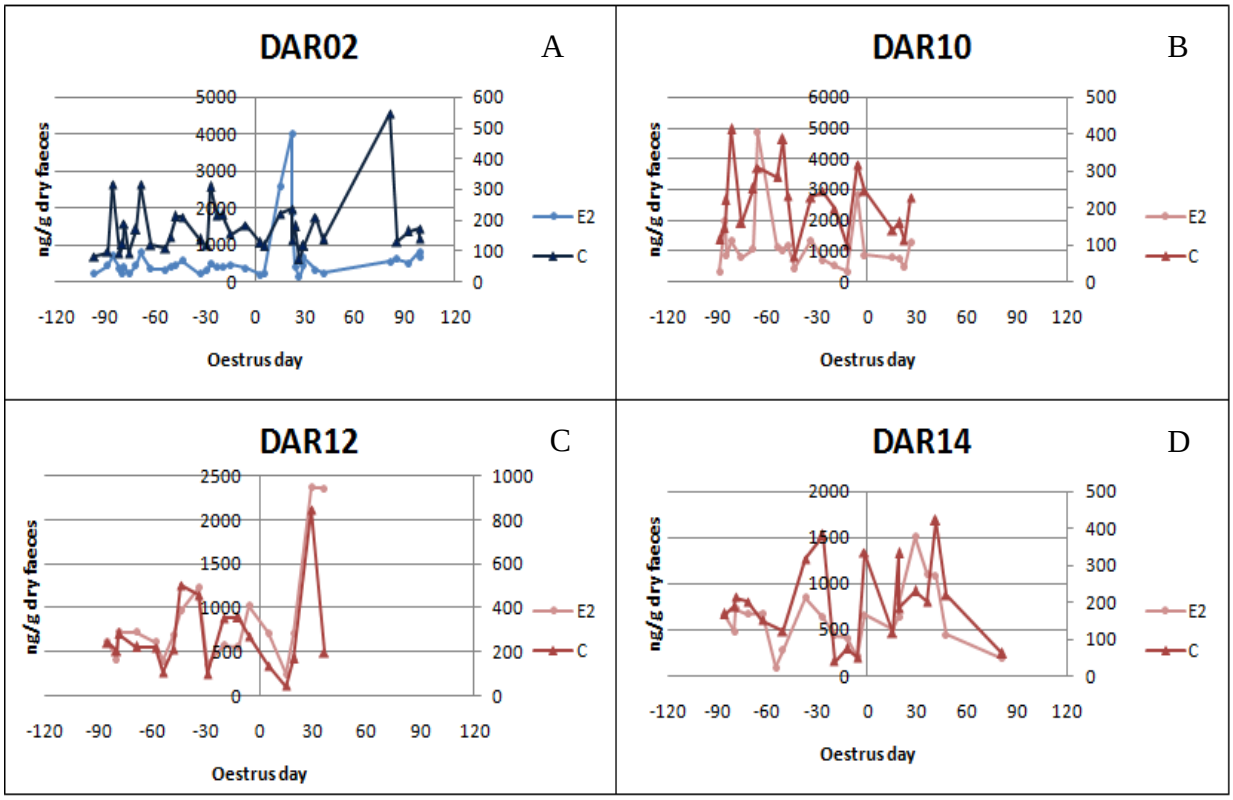

Figure 4.23 Cortisol (C) and oestradiol (E2) in the Darkeena females (dominant female DAR02 and subordinate females DAR10, DAR12 and DAR14). Note that all four females disappeared in the 2008-09 rabies epizootic.

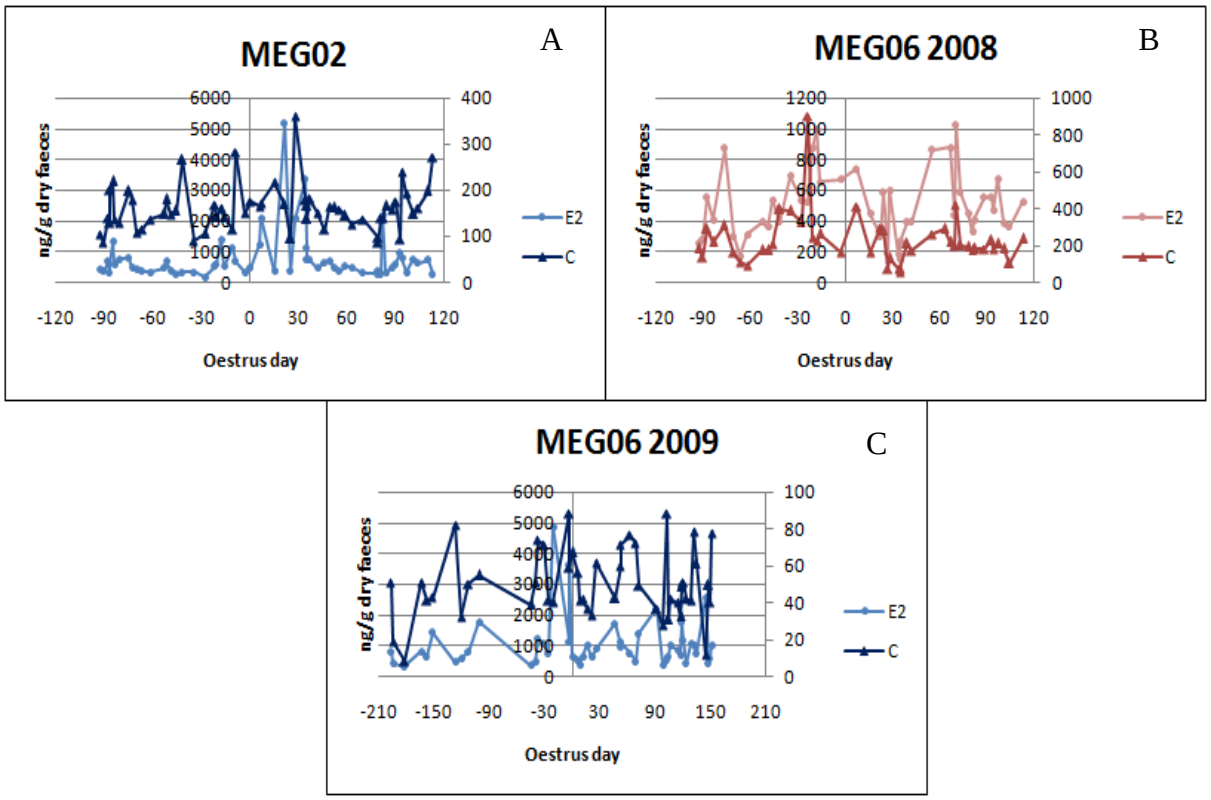

Figure 4.24: Cortisol (C) and oestradiol (E2) in the Megity females (dominant females MEG02 and MEG06 in 2009 and subordinate female MEG06 in 2008).

4.4 Discussion

Oestrus: Oestradiol levels and evidence for hormonal reproductive suppression in subordinate females

Based on behavioural observations and timing of birth, oestrus was estimated to last 15 days in Ethiopian wolves, a figure that is consistent with domestic dogs (5-15 days, Jöchle & Andersen, 1976), and comparable with grey wolves (average of 9 days, Seal et al., 1979) and coyotes (average of 10.3 days, Kennelly & Johns, 1976). Frozen samples yielded oestradiol concentrations between 128 and 856 ng/g faeces. These values are consistent with the range found in domestic bitches (Gudermuth et al., 1998) and maned wolves (Songsasen et al., 2006).

We found significant variability between oestradiol levels of individual females. Variability in oestradiol levels has also been reported for red wolves and domestic bitches. Walker et al. (2002) found that, of six females showing oestradiol peaks, peak values ranged from 169.9 to 1032.9 ng/g. Similarly, peak oestradiol levels varied in domestic Beagle bitches, with peaks ranging from 130-850 ng/g (Gudermuth et al., 1998). These studies show that individuals can show great variation in oestradiol levels, even in domestic bitches of the same breed. Due to this variability, we found that the best way to objectively define an oestradiol peak was as an increase in oestradiol from baseline levels, rather than as a fixed concentration of oestradiol per gram of faeces, and set the threshold at a 2.6 fold increase over baseline values. Gudermuth et al. (1998) found faecal oestradiol increases at oestrus in domestic bitches to range between 2.2 and 14.2 fold over anoestrus (baseline) values, and in grey wolves serum oestradiol increases at oestrus ranged from 1.5 to 14 fold increase over anoestrus (baseline) values. These studies show that an increase

between 2.6-9.4 fold, as found in this study, falls within the range of oestradiol increases at oestrus seen in other canids.

The majority (29 out of 31) of observed mating behaviours involved dominant females, which is consistent with earlier findings (Sillero-Zubiri et al., 1996a). Dominant females were approached and mounted by subordinate males in their own packs on five observed occasions. In each case the dominant female seemed receptive and stood tail aside, although in three of these cases the dominant male chased the subordinate males away from the dominant female. These observations are consistent with those found by Sillero-Zubiri et al. (1996a). Dominant females seem to exercise control over whom they mate with. For instance, we observed DAR05 trying to mate with MEG02. MEG02 was unreceptive to DAR05, although she mated with two other males just hours after DAR05 attempted to mount her. Although dominant females may be receptive to subordinate males in their pack, the dominant male will try to prevent mating.

Three surviving subordinate females showed physical signs of (pseudo)pregnancy, including an extended abdomen and/or lactation. In some canid species, such as red foxes, infanticide is common (Braastad & Bakken, 1993; Macdonald, 1980), and Sillero et al. (1996) speculated that a dominant female Ethiopian wolf killed a subordinate's litter. Since pregnancy and pseudopregnancy are difficult to distinguish in canids, it is difficult to know whether subordinates were pregnant but lost their litters, or were pseudopregnant. However, since no subordinate females were observed tied with males, and none showed physical signs of having given birth, we believe that these females were pseudopregnant and not pregnant. Subordinate female Ethiopian wolves rarely breed successfully: Randall

et al. (2007a) found that 48 out of 49 pups in 12 litters could be assigned to the pack's dominant female, with only one pup assigned to a subordinate female.

Subordinate females had lower oestradiol levels than dominant females between oestrus days -5 and +20, and none had an oestradiol peak, nor showed oestrus behaviour during this time. In contrast, all of the dominant females showed an oestradiol peak and/or oestrus behaviour including mating between days -5 and +20. These results suggest that subordinate female Ethiopian wolves are hormonally reproductively suppressed during the annual mating season. Further evidence for hormonal reproductive suppression is provided by MEG06. During the 2008 mating season, when MEG06 was subordinate to dominant female MEG02, MEG02 showed an oestradiol peak, but MEG06 did not. During the 2009 mating season, however, when MEG06 was dominant, she showed oestradiol peaks of the same magnitude as MEG02 had shown in the previous year, and mated, became pregnant and gave birth. These data suggest that it was the presence of MEG02 that previously reproductively suppressed MEG06 and prevented her from breeding.

It is interesting to note that two subordinate females and one previously subordinate female did show signs of oestrus both through oestradiol peaks and through oestrus behaviour. SOD06, originally a subordinate female in Sodota pack, was seen mating a month after dominant female SOD02 came into oestrus. She appeared to have become pregnant and split from her original pack with male SOD07, although she failed to rear pups. DAR12 was seen standing tail aside for DAR03, the pack's dominant male, on oestrus day 27, as dominant female DAR02 looked on. Similarly, female BBC42 was also seen mounted by a male on oestrus day 47. Previous observations also suggest that subordinate females may show mating behaviours outside of the annual mating season. Sillero-Zubiri (1994)

observed two subordinate females engaging in pre-mating behaviour, but these observations were made later than the annual mating season (C. Sillero-Zubiri, pers. comm.). Similarly, in Sodota pack in 1990, a subordinate female became pregnant approximately one month later than the dominant female. This subordinate female tried to establish a new pack on the boundary of the original pack territory and gave birth. However, it seemed that the dominant female killed the subordinate female's pups and the subordinate female, as well as the other wolves who had tried to leave the original pack, rejoined Sodota pack and helped at the dominant female's den (Sillero-Zubiri et al., 1996a). These observations show that some subordinate females may come into oestrus outside of the annual mating season. This may be a factor in determining whether females stay in their natal pack, or split to create a new pack. Although rare, cases of subordinate females becoming pregnant and staying in the pack have also been reported. For example, Randall et al. (2007b) found that one of 49 pups could be attributed to a subordinate female. Similarly, a litter containing pups of different ages (presumably from pregnancies at different times in the dominant and subordinate female) has been observed in the Web Valley previously (J. Marino, pers. comm.) These data suggest that, although most subordinate females are reproductively suppressed during all of the mating and breeding season, some subordinate females may ovulate and attempt opportunistically to breed.

Although patterns emerged when data were combined, we did not detect an oestradiol peak in all females that came into oestrus. Female SOD02 in 2009 did not show a clear oestradiol peak at oestrus, although oestrus was confirmed by observed mating, pregnancy and the emergence of pups. It may be that we missed SOD02's oestradiol peak due to an irregular sampling frequency. Although SOD02 was sampled on average every 3.5 days

between oestrus days -50 and +150, there was a six day period between days +4 and +10 when no samples were collected from SOD02.

Several oestradiol peaks were also found outside of the annual mating season. Females SOD02 in 2009, NYA36, BBC32 and DAR02 in 2007 had oestradiol peaks during their pregnancies, on oestrus days 60, 36, 59 and 58 respectively. Oestradiol peaks during pregnancy have also been observed in maned wolves (Wasser et al., 1995) and domestic bitches, and are presumably of luteal origin (Concannon et al., 1977; Concannon et al., 2009). Subordinate female DAR10 showed a large oestradiol peak on oestrus day -66, but we did not observe any mating behaviours and DAR10 did not become pregnant. Subordinate female DUM04 also showed oestradiol peaks on oestrous days 46, 84 and 130, but was not observed showing any mating behaviours. These oestradiol peaks cannot be easily explained, although we cannot exclude the possibility that DAR10 and DUM04 did come into oestrus outside of the annual mating season, but that we failed to observe any oestrus behaviour. Domestic bitches may undergo a 'silent heat', which is not characterized by the usual signs of oestrus, and can easily be mistaken for anoestrus (Okkens et al., 1992). It is possible that some of our subordinate females had a silent oestrus when they showed oestradiol peaks but we failed to observe signs of oestrus.

Pregnancy and pseudopregnancy in females

Frozen samples yielded progesterone concentrations between 132 and 3869 ng/g faeces. This is higher than the faecal progestin concentrations found in domestic dogs (peak progesterone concentrations between 125-1340 ng/g faeces, Gudermuth et al., 1998), but lower than faecal progestin levels found in maned wolves (levels ranging from 1.7-68.8

micrograms/g faeces, Songsasen et al., 2006). Based on observations of mating and birth of pups, pregnancy was estimated to last about 60 days, which is consistent with previous estimates (Sillero-Zubiri & Gottelli, 1994) as well as pregnancy duration in closely related grey wolves (63 days, Seal et al., 1979).

On average, both dominant pregnant and subordinate non-pregnant females had higher progesterone levels between pregnancy days -5 to +65. Progesterone levels of dominants and subordinates did not differ significantly between days -5 to +65 nor on the other days during the field season. These results suggest that subordinate females often become pseudopregnant during their dominant female's pregnancy. In many canids, pseudopregnancy is difficult to distinguish from pregnancy. For example, both pregnant and pseudopregnant domestic bitches show increases in progesterone levels (Concannon et al., 1975), and pseudopregnant bitches may also show physical signs of pregnancy such as an extended abdomen and lactation (Chakraborty, 1987). However, as pseudopregnancy can be overt or covert (Smith & McDonald, 1974), pseudopregnant females may also lack any physical traits of pseudopregnancy. Three subordinate females in this study showed physical signs of pregnancy such as an extended abdomen and/or allosuckled the pups. However, the fact that no subordinate female was seen tied with a male, and showed no signs of having given birth, suggests that they were pseudopregnant and not pregnant.

Pseudopregnancy is thought to cause spontaneous lactation, which is thought to increase the inclusive fitness of allosuckling females, who may improve the survival rates of offspring closely related to them (Creel et al., 1991). Since pseudopregnancy can also result in mothering behaviour (Chakraborty, 1987; Concannon et al., 2009), the potential evolutionary advantages of pseudopregnancy in cooperative breeders are clear. Of the five

surviving subordinate females in our study, four were in a pack where pups emerged and two of these four females allosuckled the pups. This number is comparable with previous studies by Sillero-Zubiri and Gotteli (1994), who found that 8 of 18 dens watched had an allosuckling subordinate female. In Ethiopian wolves, dens with allosuckling females were found to have smaller litters at emergence, but allosuckling did increase post-emergence survival of pups (Sillero-Zubiri, 1994). Our dataset only includes two dens which had allosuckling females (Sodota and Dumal packs in 2009), so it is difficult to make comparisons on reproductive success between dens that did and did not have allosuckling females, especially as this study was concluded when the allosuckled pups were about three months old, so the longer term survival was not determined.

Despite the fact that seven of the nine subordinate females in this study had increased levels of progesterone between pregnancy days -5 to +65, and/or physical signs of pseudopregnancy, none of the subordinate females showed an oestradiol peak or oestrus behaviour during the annual mating season. Female DAR12 came into oestrus around day +27, when she was seen standing tail aside for a male. DAR12 also showed a fourfold increase in oestradiol levels at this time. Females DAR10 and DAR14 were not observed in any oestrus behaviour but did show oestradiol peaks on oestrus days -6 and +29 respectively, indicating they may have come into oestrus outside the annual mating season. Unfortunately, DAR10, DAR12 and DAR14 disappeared in the 2008-09 rabies epidemic, so we cannot be sure if they were pseudopregnant or pregnant.

Four subordinate females (DAR08, MEG06 in 2008, SOD04 and DUM04) became pseudopregnant despite never having an oestradiol peak more than twofold over baseline values. Pseudopregnancy in canids is the result of a non-conceptive oestrous (e.g.

Chakraborty, 1987; Creel et al., 1997b; Concannon et al., 2009). Increased progesterone levels in pseudopregnant domestic bitches are thought to be produced by the corpus luteum following ovulation (Hoffmann et al., 2004). Although in other species progesterone may also be produced by the placenta (e.g. humans, see Hadley, 2000) or the adrenal gland (e.g. white tailed deer, *Odocoileus virginianus*, Plotka et al., 1983), in domestic bitches the placenta has not been found to be a source of progesterone (Olson et al., 1984; Smith & McDonald, 1974; Concannon et al., 2009) and Smith and McDonald (1974) found no evidence to indicate any increase in adrenal progesterone during pregnancy. For this reason, it is unusual that we saw evidence of pseudopregnancy in four females who appeared to be acyclic.

It is possible that subordinate females SOD04, MEG06, DAR08 and DUM04 did show oestradiol peaks but that we missed them. Despite a frequent average sampling frequency for females SOD04 and MEG06 in 2008 (sampled on average every 4.9 and 4.6 days respectively), both females had periods of more than seven days when they were not sampled. The same is true for females DAR08 and DUM04, who were sampled less regularly (sampled on average every 7.7 and 7.1 days respectively). It is therefore possible that these females did come into oestrus at some point during the field season although we failed to detect it in either our samples or behavioural observations.

Some authors (e.g. Hoffmann et al., 2004; Smith & McDonald, 1974) found that the luteal phase lasts longer in pseudopregnant domestic bitches (80 days) than in pregnant bitches (60-65 days), and Mondain-Monval et al (1977) found that in pseudopregnant red foxes the luteal phase lasted between 60-85 days, whereas pregnancy lasted 51-54 days. In domestic cats, however, the luteal phase is shorter in pseudopregnant cats when compared with

pregnant cats (Verhage et al., 1976). It may be that pseudopregnancy in Ethiopian wolves is longer or shorter than pregnancy. If this is the case, subordinate females could come into oestrus at a different time than their dominant females and still end their pseudopregnancy (including possible lactation) around the time the dominant female's pups are born. It may be that the dominant female reproductively suppresses the subordinate females during the annual mating season, but not at other times. The three subordinate females who we are sure came into oestrus did so on oestrus days +27 (DAR12), +40 (SOD06 in 2007) and +43 (BBC42). Some canids including male maned wolves (Velloso et al., 1998) and male African wild dogs (Johnston et al., 2007) show seasonal trends in reproductive parameters such as spermatogenesis, and are less fertile or even aspermic outside of the mating season. If the same is true for Ethiopian wolves, this could explain why dominant females do not invest in reproductively suppressing subordinate females outside the annual mating season, and why DAR02 did not interfere when DAR14 solicited the pack's dominant male. However, this theory could not be conclusively tested in the wild population studied here (see also Chapter 5).

In three dominant females (MEG06 in 2009, SOD02 in 2009 and DUM02) we failed to detect a clear increase in progesterone during pregnancy, although the timing of pregnancy was reliably estimated through observed mating and birth and emergence of pups from all three females. Although all three females did show higher progesterone levels during pregnancy than post-partum, they also each showed large increases in progesterone starting well before the start of pregnancy. A possible explanation could be that these females experienced a split oestrus. Split oestrus is sometimes recorded in domestic bitches and can be described as an abnormally short duration of pro-oestrus or oestrus (Meyers-Wallen, 2007), which ends before ovulation (Okkens et al. 1992). During a split oestrus,

progesterone levels may rise, although usually not as much as during a complete oestrus (Meyers-Wallen, 2007). A split oestrus may be followed by a complete oestrus within weeks or even days, which may explain the patterns seen in DUM02, SOD02 and MEG06. Subordinate females NYA32 and BBC42 also did not show a clear increase in progesterone between days -5 and +65. Female NYA32 showed a single progesterone peak on pregnancy day -16, and her progesterone levels remained low throughout the rest of the field season. We did not observe any oestrus behaviour or an oestradiol peak in NYA32 and she did not show any overt signs of pseudopregnancy. NYA36 lost her litter, and NYA32 did not allosuckle any pups. Female BBC42 shows an increase in progesterone levels between pregnancy days -26 and +6, after which progesterone levels drop sharply and start to increase again on day +41. Female BBC42 showed signs of oestrus (including an oestradiol peak and mating behaviour) 13 days before the second progesterone peak on pregnancy day +41, so this progesterone peak may have been following an unfertile ovulation. BBC42 showed no overt signs of pseudopregnancy and did not allosuckle the pups.

Dominance rank, aggression, cortisol and reproduction

By analyzing behavioural data and cortisol levels, we tried to assess if there was a link between status, aggression and cortisol levels. We found that most aggression in wolves is targeted at neighbouring packs, rather than at members of one's own pack, and seems to be related to territory defence. Aggression between packs over territory boundaries is well documented in Ethiopian wolves (Sillero-Zubiri & Gottelli, 1994). Dominant females were more likely than subordinate females do be involved in inter-pack aggression.

Very few incidences of intra-pack aggression between females were recorded during this study, and, in one case a subordinate female (DAR12) was seen soliciting the pack's dominant male (DAR03) in front of the pack's dominant female (DAR02), without the dominant female interfering. Although anecdotal, these observations suggest that reproductive suppression in subordinate females is not regulated through aggressive behaviours. However, Sillero et al. (1996a) found that subordinate females are likely to be harassed by dominant females. Data collected by EWCP between 1988-2010 also suggests that subordinate females are much more likely to be on the receiving end of intra-pack aggression than dominant females. In addition the lowest ranking females may be subjected to aggression not only from the dominant female but from any higher ranking female (see also Sillero-Zubiri et al., 1996a). The fact that lowest ranking females may receive aggression from all higher ranking females, as well as from some males may explain why subordinate females had higher average cortisol levels than dominant females.

Despite the fact that subordinates had higher cortisol levels than dominants, it is unlikely that reproductive suppression in female Ethiopian wolves is mediated mainly through stress hormones since this difference was not significant either overall or during oestrus. In addition, cortisol levels were not consistently higher in subordinates than dominants. For instance, MEG06's average cortisol levels did not change significantly (paired T-test, $p=0.954$) after she changed from subordinate to dominant status. However, MEG06's oestradiol levels did change significantly (paired T-test, $p<0.005$) when she became dominant, indicating that cortisol was not the main method for MEG06's earlier reproductive suppression.

Our results indicate that reproductive suppression in Ethiopian wolves is exercised by the dominant female through cues that are unrelated to aggression and stress hormones, possibly pheromones (see Hradecky, 1985). Dominant females seem to suppress subordinates' oestradiol levels during the annual mating season, which prevents them from ovulating. A similar result was found by Barret et al. (1990), who found that when subordinate female marmosets were removed from their dominant female, they continued to be reproductively suppressed for a month when exposed to their dominant female's scent, but ovulated after ten days of being removed if not exposed to their dominant female's scent. However, in marmosets, dominant females have higher cortisol levels than subordinate females (Saltzman et al., 1998), indicating that reproductive suppression in marmosets is not mediated through stress hormones. Similarly, in African wild dogs, subordinate females had lower oestrogen levels than dominant females during mating periods, although dominant females had higher glucocorticoid levels than subordinates (Creel et al., 1997a).

Cortisol and oestradiol were correlated for subordinate females DAR10, DAR12, DAR14 and MEG06 in 2008, but not for dominant females DAR02, MEG02 and MEG06 in 2009. Cortisol and oestradiol levels have been found to be related in female marmosets, but in this species subordinate, acyclic females show both lower oestradiol and lower cortisol levels than dominant, cycling females (Saltzman et al., 1998). Although cortisol and oestradiol levels may be related, cortisol levels are likely to be influenced by other stressful events. In tamarins, for example, cortisol levels showed greater responses to social disruption (i.e. removing a female from her social group) than to regular reproductive cycling (Ziegler et al., 1995). Patterns of cortisol and oestradiol are similar in dominant females DAR02, MEG02 and MEG06 but each of these females show several high cortisol

values that do not correspond with high oestradiol values (e.g. DAR02 on oestrus day 81, Fig. 4.23A, MEG02 on oestrus day 113, Fig. 4.24A). These high values probably correspond with stressful events that occurred hours before sample collection. Unfortunately we cannot explain specific cortisol peaks from our behavioural data, as the events triggering the cortisol peaks would have happened the day before the sample was collected due to the time delay between blood and faecal hormones (see also Palme et al., 1996). As packs were generally tracked every two or three days, we do not have consecutive daily behavioural observations. In summary it appears that oestradiol and cortisol are generally correlated in female Ethiopian wolves, but sudden cortisol peaks (presumably caused by stressful situations) may occur.

Although in domestic dogs cortisol levels increase just before parturition (Concannon et al., 1978), we did not see any evidence of this in Ethiopian wolves (Fig. 4.22). However, Concannon et al. (1978) took serum samples two or three times daily from domestic bitches, so it may be that our sampling frequency was too low to detect a rise in cortisol just before parturition.

Chapter 5: Sex, stress and social status: patterns in testosterone and cortisol in male Ethiopian wolves[2]

[2]A version of this chapter has been prepared for submission to Hormones and Behavior as: van Kesteren et al, Sex, stress and social status: patterns in testosterone and cortisol in male Ethiopian wolves

Abstract

Ethiopian wolves, *Canis simensis*, live in family packs and breed cooperatively. Within a pack, mating and breeding is monopolized by the dominant male and female, although extra-pack copulations are common, and subordinate males may sire pups in neighbouring packs. Faecal samples were collected regularly from nine male Ethiopian wolves, and opportunistically from fourteen male Ethiopian wolves. These samples were analyzed for testosterone and cortisol using radio immunoassays (RIA). We tested the predictions of the Challenge Hypothesis, namely that testosterone levels would be higher during times of mating and increased aggression (which coincide in Ethiopian wolves) and lower when there were pups in the pack to care for, as all Ethiopian wolves in a pack contribute to the rearing of pups. No clear seasonal pattern was detected in testosterone levels, indicating that Ethiopian wolves do not conform to the predictions made by the Challenge Hypothesis. Similarly, no seasonal patterns were found in male levels of cortisol, although regularly collected samples showed that dominant males had higher average testosterone and cortisol levels than subordinates. Our conclusions are consistent with previous findings in African wild dogs, *Lycaon pictus*, and grey wolves, *Canis lupus*, and may be related to higher rates of aggression and mate guarding in dominant males.

5.1 Introduction

Canid reproductive biology often includes cooperative breeding, reproductive suppression of subordinates, pseudopregnancy and alloparental care (Asa & Valdespino, 1998). For example, in cooperatively breeding canids such as African wild dogs, *Lycaon pictus* (Creel et al., 1997a), breeding is largely monopolized by the dominant pair, and subordinate pack members help to rear the dominant pair's pups. Most wild canids are seasonal breeders, with one breeding season per year (Chapter 2), and males often show seasonal patterns in

testosterone levels and/or other reproductive parameters such as testicular volume. For example, in coyotes, *Canis latrans*, serum testosterone, testicular volume, ejaculate volume and sperm concentration all peak during the mating season (Minter & DeLiberti, 2008), and in male Arctic foxes, *Alopex lagopus*, androgens peak during the mating season (Smith et al., 1985). In other seasonally breeding canids including maned wolves, *Chrysocyon brachyrus* (Velloso et al., 1998), and African wild dogs (Creel et al., 1997a; Johnston et al., 2007), no seasonal patterns in testosterone were found, despite both species showing seasonal trends in reproductive parameters such as testicular volume and spermatogenesis.

Ethiopian wolves share many of the reproductive features described by Asa and Valdespino (1998). They live in packs of between two to eight adults, one to six yearlings and up to six pups (Marino et al., 2006; Sillero-Zubiri et al., 1996a). Within a pack, there is a social hierarchy, with a single dominant breeding pair (Sillero-Zubiri & Gottelli, 1994). However, subordinate males may try to mate with dominant females in neighbouring packs (Sillero-Zubiri et al., 1996a, Chapter 4) and extra-pack paternity in litters has been found (Randall et al., 2007; Gottelli et al., 1994). Male Ethiopian wolves tend to be incorporated into the pack as adults, whereas females are more likely to disperse (Sillero-Zubiri et al., 1996a). All pack members help to rear the pups through den guarding, regurgitating prey to the pups, and subordinate females may also allosuckle the pups (Sillero-Zubiri et al., 1996a). Ethiopian wolves, like most other canids such as coyotes (Kennelly & Johns, 1976), and grey wolves, *Canis lupus* (Seal et al., 1979), are seasonal breeders, with a single breeding season per year (Sillero-Zubiri et al., 1998; Sillero-Zubiri et al., 1996a).

In some cooperative breeders, differences in the testosterone levels of dominant and subordinate males have been found. For example, in African wild dogs, dominant males mate more effectively (Creel et al., 1997a), father most pups (Girman et al., 1997) and have higher testosterone levels than subordinate males (Johnston et al., 2007). However, subordinate males in cooperatively breeding species do not always have lower testosterone levels, as is the case for red-cockaded woodpeckers, *Picoides borealis*, where male breeders and helpers have equivalent plasma testosterone concentrations (Khan et al., 2001). Similarly subordinate male dwarf mongooses, *Helogale parvula*, have testosterone levels which are indistinguishable from testosterone levels in dominant males (Creel et al., 1992).

Cooperative breeding requires reproductive suppression of subordinates, and one way in which subordinate animals may be reproductively suppressed is through stress hormones. Subordinate animals may be stressed as a consequence of being subordinate, and thus have higher levels of glucocorticoids, which in turn can lower an individual's testosterone levels (Blanchard et al., 2001). Although early studies in captive animals indicated that subordinates had higher glucocorticoid levels, more recently it is thought that in the wild, subordinates may more easily avoid aggression, whilst dominants have to behave aggressively to maintain their status, leading to higher glucocorticoid levels (for a review see Creel, 2001). For example dominants of both sexes had higher glucocorticoid levels than subordinates in cooperatively breeding African wild dogs (Creel et al., 1997a) and grey wolves (Sands & Creel, 2004). An alternative hypothesis is that subordinates may be behaviourally rather than hormonally suppressed. For example, although male subordinate dwarf mongooses, *Helogale parvula*, do not have lower testosterone levels than dominant

males, they are prevented from mating by aggression from the dominant male (Creel et al., 1992).

As well as playing an important role in reproduction, testosterone is also linked to other behaviours, especially aggression (Delvonne et al., 1996). The link between testosterone and aggression has been well studied in several species. Experiments in birds have shown that castration tends to decrease the frequency of aggression, and replacement therapy with testosterone tends to increase it (reviewed in Harding, 1981). Similarly, in male ring tailed lemurs, *Lemur catta* (Cavigelli & Pereira, 2000), and chimpanzees, *Pan troglodytes schweinfurthii* (Muller & Wrangham, 2004), faecal testosterone levels correlated positively with aggression during the mating season.

One theory that outlines the expected relationship between season, aggression and testosterone in seasonal breeders is the Challenge Hypothesis, coined by Wingfield et al. (1990). Briefly, the Challenge Hypothesis states that, as the breeding season begins, testosterone levels rise from a non-breeding baseline to a slightly higher breeding baseline, stimulated by environmental cues such as day length. Breeding baseline levels of testosterone are sufficient for normal reproductive physiology such as spermatogenesis and reproductive behaviour. Temporal patterns in testosterone are then predicted to differ between species, based on the amount of paternal care and the type of breeding system. Species that show high levels of paternal care are expected to show lower levels of testosterone than polygynous species, and levels of testosterone are predicted to be lower when there are young to care for. The Challenge Hypothesis also predicts that increases in testosterone above breeding baselines are related to aggressive behaviours, such as maintenance of a breeding territory or mate guarding (Wingfield et al., 1990).

Although the Challenge Hypothesis is based mostly on data from birds, it has been tested in some mammalian species. One experimental evaluation of the Challenge Hypothesis in mammals involved wild dwarf mongooses (Creel et al., 1993). In dwarf mongooses, only the dominant female breeds and male care for offspring is common. Given this breeding system the prediction is that androgen levels in dwarf mongooses should be elevated above the breeding baseline only during periods of mating or aggression (which coincide in dwarf mongooses). However, urinary androgen levels did not increase during periods of mating or aggression, nor decrease when there were young to care for. In chimpanzees however, the predictions of the Challenge Hypothesis were mainly upheld. Male chimpanzees showed significant increases in testosterone levels during the time that parous females showed maximally tumescent sexual swellings, periods which were also marked by increased rates of male aggression. In addition, the highest ranking male chimpanzee showed the highest levels of urinary testosterone (Muller & Wrangham, 2004). Similarly degus, *Octoden degus*, were found to conform to the predictions of the Challenge Hypothesis, and had increased testosterone levels during periods of increased agonistic interactions (Soto-Gamboa et al., 2005).

Although some aspects of Ethiopian wolf reproduction, such as breeding system (Sillero-Zubiri et al., 1996a), seasonality (Sillero-Zubiri et al., 1998) and paternity (Randall et al., 2007), have been studied before, nothing is known about the reproductive physiology of male Ethiopian wolves. Here we use faecal samples collected from wild male Ethiopian wolves to describe their physiology and answer the following questions:

1. Are there any seasonal trends in testosterone levels in male Ethiopian wolves?

   Ethiopian wolves are seasonal breeders, with only one mating season a year (Sillero-Zubiri et al., 1998). A seasonal pattern in testosterone would therefore be expected, as has been observed in several other canid species (Chapter 2). The Challenge Hypothesis (Wingfield et al., 1990) predicts that testosterone levels should be higher at times of mating and/or aggression, which coincide in Ethiopian wolves (Sillero-Zubiri & Gottelli, 1994; Sillero-Zubiri & Macdonald, 1998), and lower during pup rearing times, as all male Ethiopian wolves in a pack provide care for the pups (Sillero-Zubiri et al., 1996a). We therefore predict that male Ethiopian wolves will show higher testosterone levels during the mating season, and lower testosterone level when there are pups to care for.

2. Are there any differences in testosterone levels between dominant and subordinate males?

   Although usually within an Ethiopian wolf pack only the dominant male will mate with the pack's dominant female, the dominant female in a pack may mate with males (either dominant or subordinate) from other packs (Sillero-Zubiri et al., 1996a). Extra-pack paternity has been found in Ethiopian wolf litters (Randall et al., 2007; Gottelli et al., 1994). This suggests that subordinate males exhibit regular reproductive behaviour and are fertile, and are not reproductively suppressed through lowered levels of testosterone. We would therefore not expect significant differences in testosterone between dominant and subordinate males.

3. Are there differences in cortisol levels between dominant and subordinate males?

   There is some evidence that subordinate female Ethiopian wolves are harassed by higher ranking females, and that this may prompt the dispersal of subordinate

females from their natal pack (Sillero-Zubiri et al., 1998). In contrast, male Ethiopian wolves are philopatric (Sillero-Zubiri et al., 1996a), and intra-pack male aggression is less likely to play a role in promoting dispersal. However, dominant males may behave aggressively to subordinate males at times to maintain their status, and to control access to the dominant female during the mating season (see also Sillero-Zubiri et al., 1996a). In addition, several studies in other communal breeders in the wild found that dominants have higher cortisol levels than subordinates (e.g. Creel et al., 1997a; Sands & Creel, 2004). We therefore predict that dominant male Ethiopian wolves will have higher cortisol levels.

5.3 Materials and Methods

5.3A Study Population

Nine focal packs were selected and over the course of three years, in addition to sampling 23 female Ethiopian wolves, we regularly sampled nine males (three dominant and six subordinate) in Addaa, Darkeena and Sodota packs between August 2007-February 2008 (Table 5.1) and opportunistically sampled fourteen males in Web Valley between August 2008 and January 2010 (Table 5.2).

Table 5.1: Male Ethiopian wolves in Web Valley included in this study from 2007-08

Pack	Male	Status
Addaa	SOD07	Dominant
Sodota	SOD01	Dominant
Sodota	SOD03	Subordinate
Sodota	SOD05	Subordinate
Darkeena	HAR15	Dominant
Darkeena	DAR03	Subordinate
Darkeena	DAR05	Subordinate
Darkeena	DAR07	Subordinate
Darkeena	DAR09	Subordinate

During the second (August 2008-March 2009) and third (August 2009-February 2010) field seasons male samples were collected opportunistically, and the rank (dominant or

subordinate) of the defecating male was usually known. A total of 59 samples were collected opportunistically from six dominant males, and 38 samples were opportunistically collected from eight subordinate males (Table 5.2). ALA07 changed status between 2008 and 2009, from being a subordinate male in Alando pack to being the dominant Megity male, replacing MEG03. Hence ALA07 is included as both a dominant and subordinate male. Two Darkeena dominant males are also included. HAR15 was the pack's dominant male initially, but was succeeded by DAR03 after his death in October 2008 from rabies.

Table 5.2: Male Ethiopian wolves in Web Valley included in this study between 2008-2010

Pack	ID	Status
Addaa	SOD07	Dominant
Darkeena	HAR15	Dominant
Darkeena	DAR03	Dominant
Mulamo	MUL03	Dominant
Megity	ALA07 (2009-2010)	Dominant
Megity	MEG03	Dominant
Alando	ALA07 (2008-2009)	Subordinate
Darkeena	DAR05	Subordinate
Megity	MEG01	Subordinate
Megity	ALA05	Subordinate
Megity	ALA09	Subordinate
Mulamo	FUL07	Subordinate
Sodota	SOD03	Subordinate
Sodota	SOD05	Subordinate

5.3B Field Methods

For a full description of the field methods refer to Chapter 1, section 1.9. Briefly, wolves were followed on foot or horseback and behavioural observations (including date and time, wolf age, sex and ID, behavioural code and location notes) were recorded every 15 minutes. Faecal samples were collected within minutes of defecation and stored in a cooler box on ice until return at the camp. At the camp, 3 grams of sample was stored at -20°C, or 3-4g was desiccated in a camper over. Samples were later shipped to Edinburgh, United Kingdom, on dry ice (frozen samples) or at room temperature (desiccated samples).

5.3C Laboratory methods

The laboratory methods are described in detail in Chapter1, sections 1.10-1.13. Briefly, in the laboratory in Edinburgh, samples were extracted by manually grinding 0.50 of wet and 0.20g of dry sample with 4ml analytical grade methanol and 0.50ml double distilled water, vortexing at 1400 r.p.m, centrifuging at 2500 r.p.m, repeating the extraction process, and mixing the two supernatants. The supernatants were dried under mild heat and nitrogen, and reconstituted in PGBS assay buffer. Samples were analyzed for testosterone and cortisol using radio immunoassays.

A total of 266 faecal samples from male Ethiopian wolves were analyzed for testosterone, of which 169 were stored and transported frozen. These 169 frozen samples were also analyzed for cortisol.

5.3D Data analysis

All results are expressed as nanograms of hormones per gram of wet or dry faeces. No comparisons were made between absolute values of testosterone and cortisol in frozen and dried samples, as dried samples generally gave higher absolute values of hormones (Chapter 3).

Dates were converted into oestrus dates, to allow comparisons between Darkeena, Sodota and Addaa packs in 2007 (Addaa pack mated and bred one month later than Darkeena and Sodota packs), and between the 2008 and 2009 breeding season. Oestrus dates were determined in several ways, such as when the first signs of mating behaviour were recorded, sightings of matings, and/or by calculating backwards from the birth of litters. The date on which oestrus was estimated to start using these methods was designated as day 0. To allow for slightly different times at which oestrus started in different females, the

'oestrus time' was chosen to be between days -5 and +20, that is, the fifteen days that we estimate oestrus to last, with five days extra both before and after for females who may have come into oestrus slightly earlier or later than we observed.

All observations of mating and aggression events were recorded. Recorded mating events included males sniffing/licking females' genitals, mounts and copulatory ties. Recorded aggressive events included wolves chasing each other and contact aggression. However, observations of aggressive events were rare. To further study patterns of aggressive events between dominant and subordinate males and females, data collected by EWCP between 1988 and 2010 was also analysed.

The frequency of aggressive events between dominants and subordinates was tested using χ^2 tests, with Yates corrections if expected values were less than 5 (Sokal &Rohlf, 1981). To ensure independence of datapoints observations relating to the same male were recorded only once, so that, for example, if one male was seen behaving aggressively several times in the same fieldseason, this was treated as one observation. Testosterone and cortisol levels between dominant and subordinate males, and between reproductive periods of the year (e.g. oestrus and non-oestrus), were tested using GLMs, blocking for individual males to correct for individual variation between males (Grafen & Hails, 2002). Before using GLMs we tested that the assumptions were met. Where necessary, responses in these models were log transformed. To compare hormone levels between dominant and subordinate males, a between subject effect in these analyses, we used a single summary approach (Grafen and Hails, 2002), using two sample T tests to compare average levels of hormones in dominants and subordinates. The level of significance was set at $p \leq 0.05$. χ^2

tests were done in Microsoft Excel® and GLM analyses and T-tests were done using Minitab® statistical software.

5.4 Results

5.4A Behavioural observations

Mating behaviour

Focal males were observed in thirteen mating events during the 2007-08 field season (Table 5.3, Appendix II). Four mating events involved the dominant male and female in a pack, four involved the dominant female and a subordinate male in a pack, and in the remaining mating events the identity and status of the male could not be reliably identified (Table 5.3).

Table 5.3: Summary of the observed mating events in Web Valley between 2007-2008 and 2009-10

Female status	Male status	# of observed mating events 2007-08	# of observed mating events 2008-10
Dominant	Dominant	4	8
Dominant	Subordinate	4	1
Dominant	From another pack/not identified	5	2
Subordinate	Dominant	0	1
Total		**13**	**12**

Between 2008 and 2010, the sampled males were also observed engaged in mating events (Table 5.3). DAR03 mounted DAR02, on November 6th 2008, although there was no copulatory tie. SOD01 mounted his dominant female, SOD02 on November 15th 2008. On 4 December 2008, dominant male DAR03 mounted subordinate female DAR12, although there was no copulatory tie. Darkeena subordinate male DAR05 mounted Megity dominant female MEG02 twice on 17 September 2009. Dominant male ALA07 sniffed dominant female MEG02's vulva and mounted her four times before becoming tied with her on 22

September 2009. Subordinate male ALA09 also mounted MEG02 on 22 September 2009, but was chased away by dominant male ALA07.

Male aggressive behaviour in inter and intra-pack interactions

During this study 32 aggressive encounters between wolves that involved at least one male were observed. A further 33 aggressive interactions involving at least one male were recorded by EWCP between 1988-2010. Of these 65 aggressive encounters, 48 were inter-pack aggressions, and 17 were intra-pack. Sixteen cases of inter-pack aggression were instigated by dominant males (alone or with other wolves), and five by the dominant male and subordinate male(s). On ten occasions a subordinate male and other wolves (females, juveniles) instigated inter-pack aggression. The status of the males involved in the remaining aggressive interactions could not be determined (Table 1).

Five intra-pack aggressions were instigated by the dominant male, and targeted either subordinate males (n=4) or a subordinate female (n=1). A further three observations related to mate guarding, and involved a dominant male chasing subordinate males away from the dominant female. On 5 September 2007, HAR15, Darkeena's dominant male chased subordinate male DAR05 and an unidentified male away from dominant female DAR02. On 9 September 2007, an adult male (probably SOD01), chased away a subadult that had been mating with dominant female SOD02. On 22 September 2009, dominant male ALA07 chased subordinate pack member ALA09 away from dominant female MEG06. Five intra-pack aggressions were instigated by a subordinate male against a lower ranking male (n=4) or subordinate female (n=1). On two occasions a dominant male stole prey from the dominant female. Dominant males were never the recipient of intra-pack aggressions. These data are limited, but suggest that dominant males are more likely than subordinate males to instigate inter-pack aggressions, although this relationship is not

significant (χ^2, p=0.12). Although dominant and subordinate males were equally likely to instigate intra-pack aggression towards other pack members (χ^2, p=0.52), subordinate males were significantly more likely than dominant males to receive intra-pack aggression (Yates corrected χ^2, p=0.008).

Table 5.4: Aggressive inter and intra-pack interactions involving males recorded in this study and by EWCP between 1988 and 2010

Inter-pack aggression	
Instigated by	**Observed events**
Dominant male alone	4
Dominant male and others (females, juveniles)	12
Dominant and subordinate male	5
Subordinate male alone	0
Subordinate male and others (females, juveniles)	10
Unidentified	17
Total	*48*
Intra-pack aggression	
Description	**Observed events**
Dominant male aggressive to subordinate male (not related to mate guarding)	4
Dominant male aggressive to subordinate male (related to mate guarding)	3
Dominant male aggressive to subordinate female	1
Dominant male steals food from dominant female	2
Subordinate male aggressive to lower ranking male	4
Subordinate male aggressive to subordinate female	1
Unidentified	2
Total	*17*

5.4B Testosterone levels relating to mating and aggression

There were no significant differences in testosterone levels between times of oestrus, non-oestrus and pup rearing times in either dominant (GLM, $F_{2,2}$=0.55, p=0.58) or subordinate (GLM, $F_{2,5}$=0.37, p=0.69) males (Fig. 5.2). However, since both DAR02 and SOD06 lost their litters in 2007, there were only three males (SOD01, SOD03 and SOD05) who had pups to rear, so data on this is limited. Dominance status, however, did significantly affect testosterone levels, with significantly higher levels overall in dominant males (single summary statistic two sample T-test, DF=5, p=0.028, Fig. 5.1). Differences during mating or during pup rearing were not significant (single summary statistic two sample T-test,

DF=2, p>0.05), but were significant during non-mating times (single summary statistic two sample T-test, DF=4, p=0.019, Fig. 5.2).

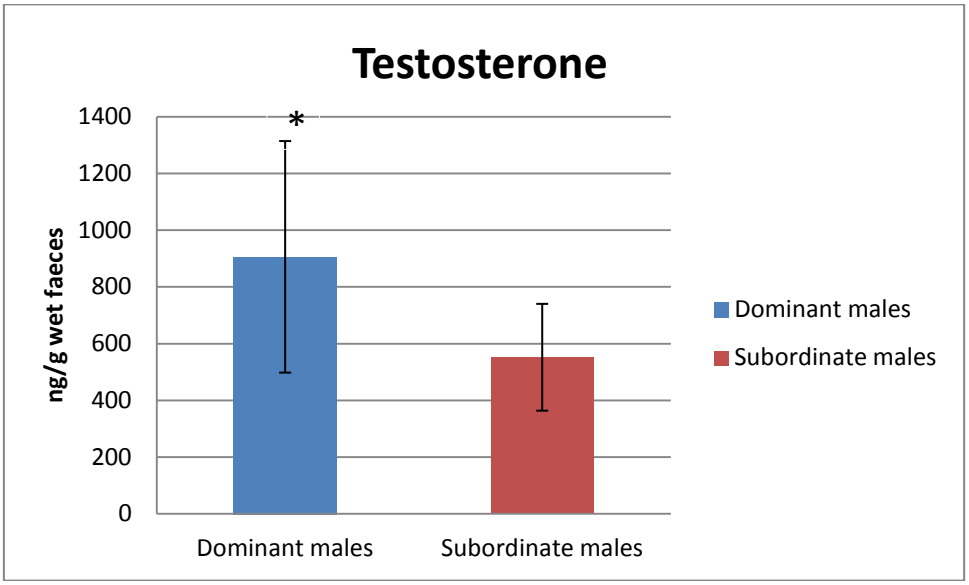

Figure 5.1: Testosterone levels of dominant (n=3) and subordinate (n=6) males over the whole fieldseason (days -23 to +145. The asterisk (*) denotes a significant difference. Error bars denote standard error of individual wolves.

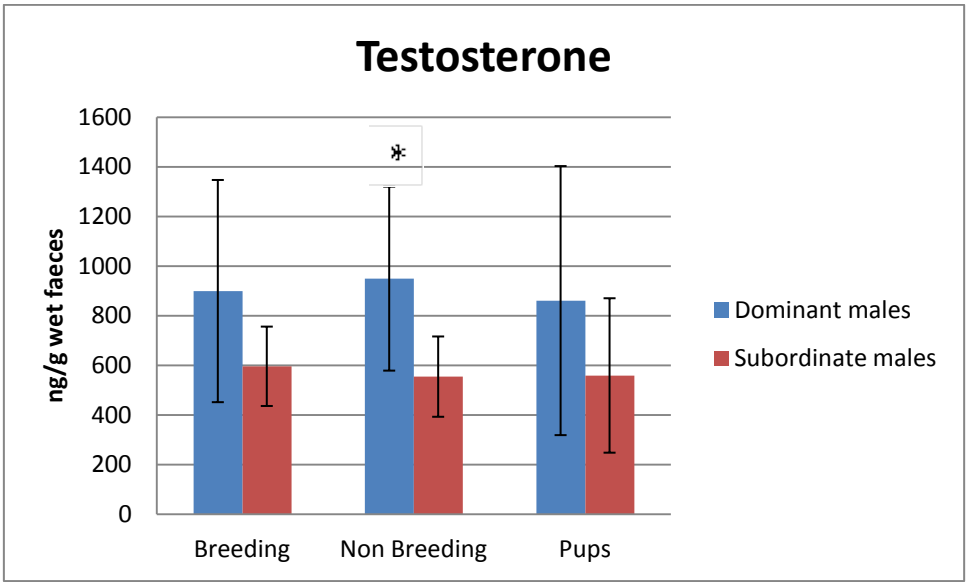

Figure 5.2: Testosterone levels for dominant (n=3) and subordinate (n=6) male Ethiopian wolves during periods of oestrus, non oestrus and pup rearing time. The asterisk (*) denotes a significant difference. Error bars denote standard error of individual wolves.

No clear seasonal patterns in testosterone levels could be detected for individual males in Sodota, Addaa and Darkeena packs (Fig. 5.3 and 5.4). Males HAR15 and DAR09 showed high testosterone levels around the time DAR02 gave birth (Fig. 5.3), but since DAR02

lost her litter there were no pups to care for in Darkeena pack in 2007. In Sodota pack, dominant female SOD02 did breed successfully and three pups emerged, but testosterone levels did not decrease after the birth of these pups (Fig. 5.4).

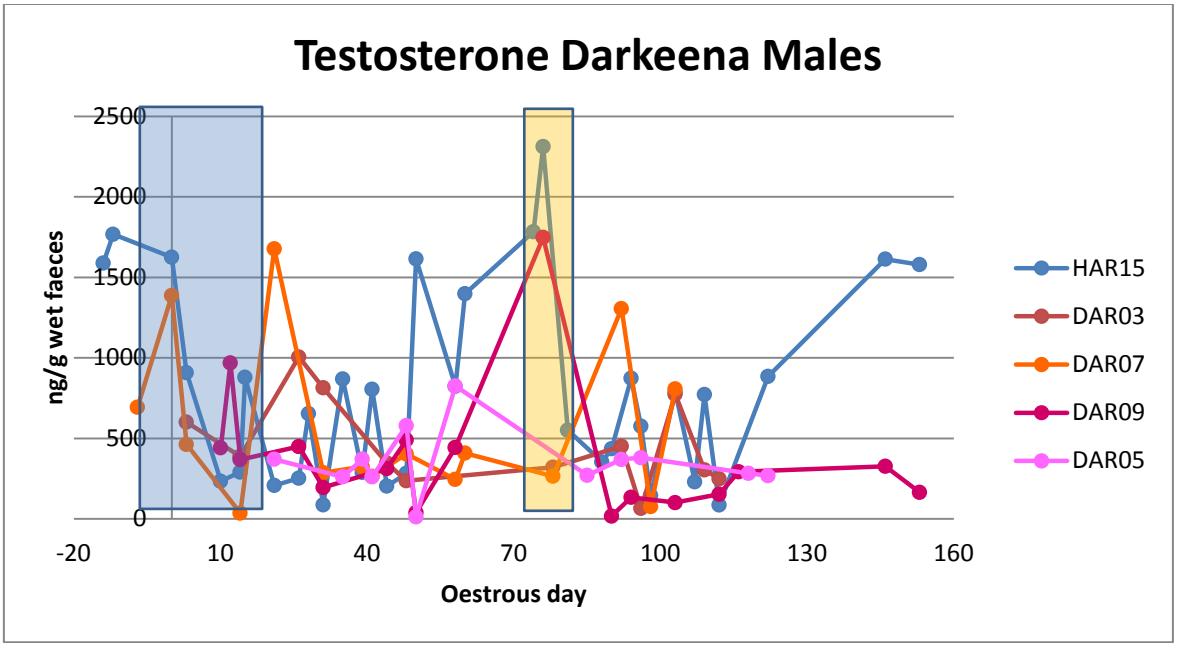

Figure 5.3: Testosterone levels in males in Darkeena (males listed in order of dominance). Blue shading area represents the time of oestrus (days -5 to +20), orange shading represents the estimated time of birth. Note that Darkeena's DAR02 lost her litter in 2007.

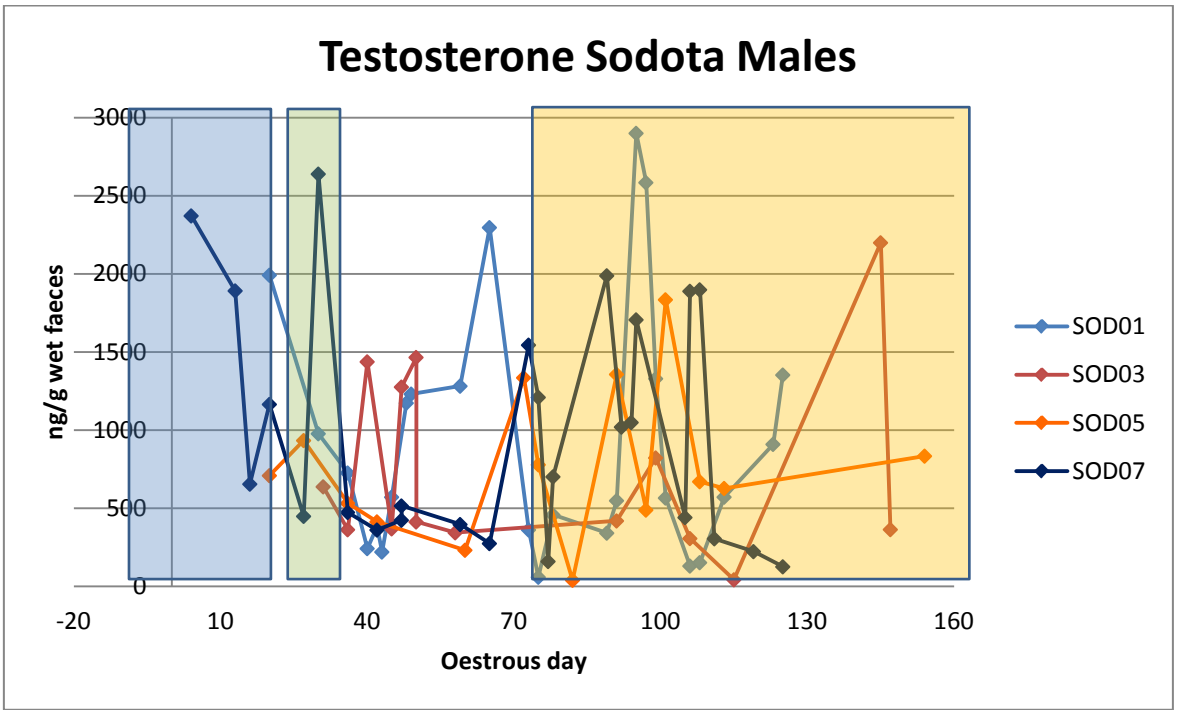

Figure 5.4: Testosterone in males in Sodota (males listed in order of dominance). Note that SOD07 is graphed together with the Sodota males, as he was originally in Sodota pack. Blue shading represents the time of oestrus (days -5 to +20), orange shading represents the pup-rearing time in Sodota pack.

Testosterone levels from opportunistically collected samples were plotted in a time series according to oestrus day (Fig. 5.5). Although two sampled dominant males (HAR15, DAR03) and two sampled subordinate males (DAR05, MEG01) died in the 2008-2009 rabies epidemic, nine of the ten surviving males were in packs in which pups emerged from the den. Testosterone levels were not significantly affected by time of year (oestrus, non-oestrus, and pup-rearing time) in either dominant (GLM, $F_{2,8}$=1.61, p=0.211) or subordinate (GLM, $F_{2,9}$=3.05, p=0.064) males. Testosterone levels of dominant and subordinate males did not differ significantly during oestrus (single summary two sample T-test, DF=8, p=0.409), nor during the pup rearing time (single summary two sample T-test, DF=7, p=0.412), but differed during non oestrus (before mating and during pregnancy), with higher levels in subordinates (single summary two sample T-test, DF=10, p=0.045, Fig. 5.6). Subordinate males had higher average levels of testosterone than dominants (Fig. 5.7), although this difference was not statistically significant (single summary two sample T-test, DF=27, p=0.907).

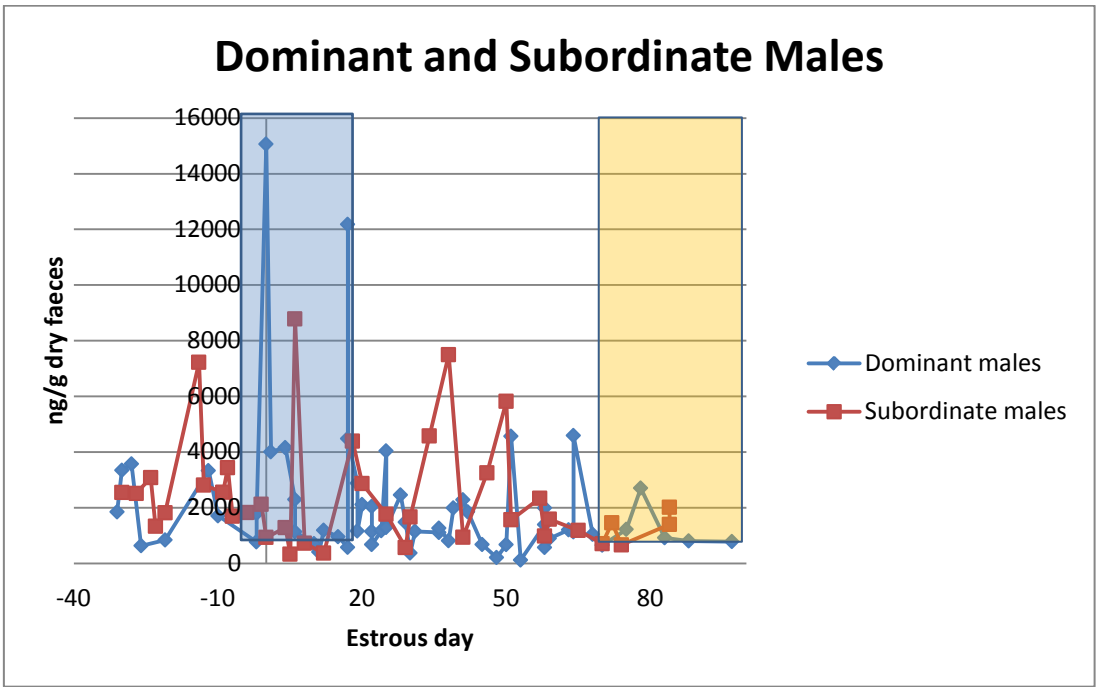

Figure 5.5: Testosterone levels for dominant and subordinate male Ethiopian wolves. Blue shading area represents the time of oestrus (days -5 to +20), orange shading represents the pup rearing time.

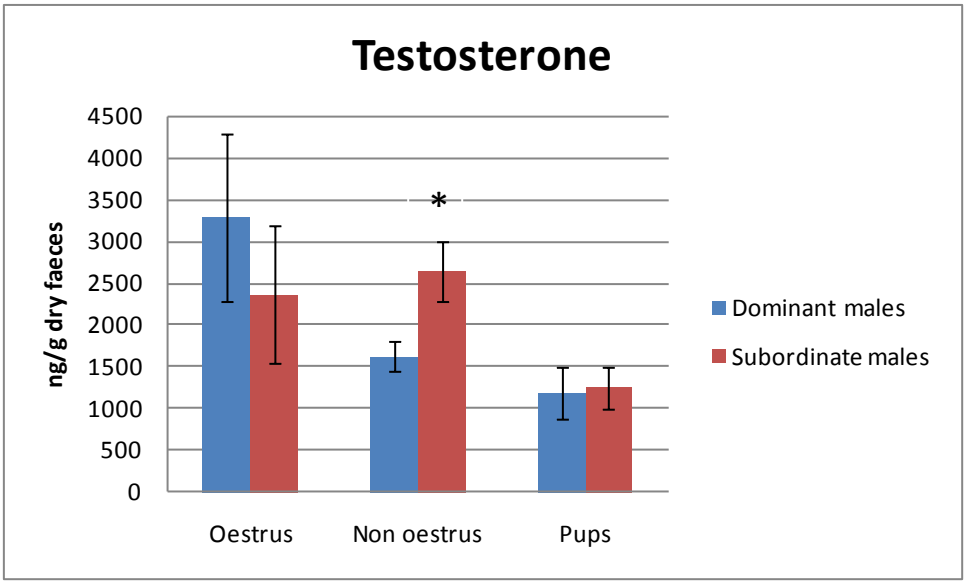

Figure 5.6: Testosterone levels for dominant and subordinate male Ethiopian wolves during periods of oestrus, non-oestrus and pup rearing times. The asterisk (*) denotes a significant difference. Error bars denote standard error of individual wolves.

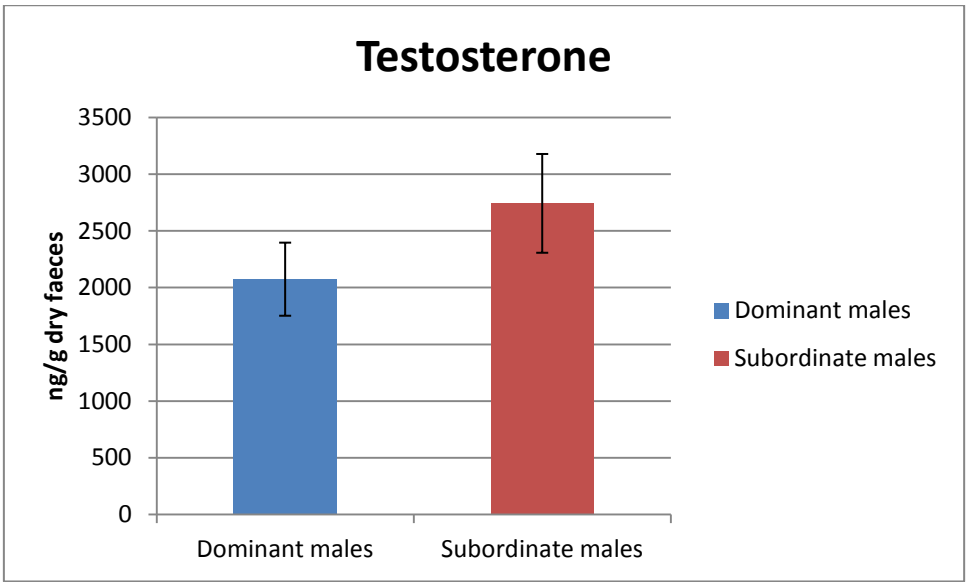

Figure 5.7: Average testosterone in dominant and subordinate male Ethiopian wolves between oestrus days -31 to +100. Error bars denote standard error of individual wolves.

5.4C Dominance rank and cortisol

Time of year (oestrus, and non oestrus) did not significantly affect cortisol levels in dominant (GLM, $F_{1,2}$=0.12, p=0.885, Fig. 5.8) or subordinate male Ethiopian wolves (GLM, $F_{1,5}$=0.14, p=0.711). Overall, dominant males had significantly higher levels of

cortisol than subordinate males (summary statistic two sample T test, DF=6, p=0.028, Fig. 5.9).

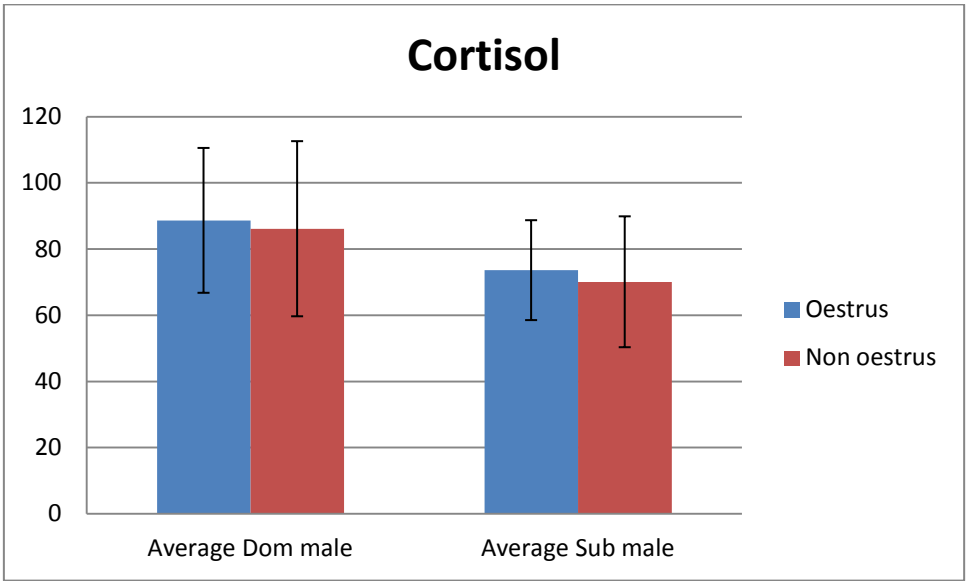

Figure 5.8: Cortisol in dominant (n=3) and subordinate (n=6) male Ethiopian wolves during oestrus and non-oestrus times. Error bars denote standard error of individual wolves.

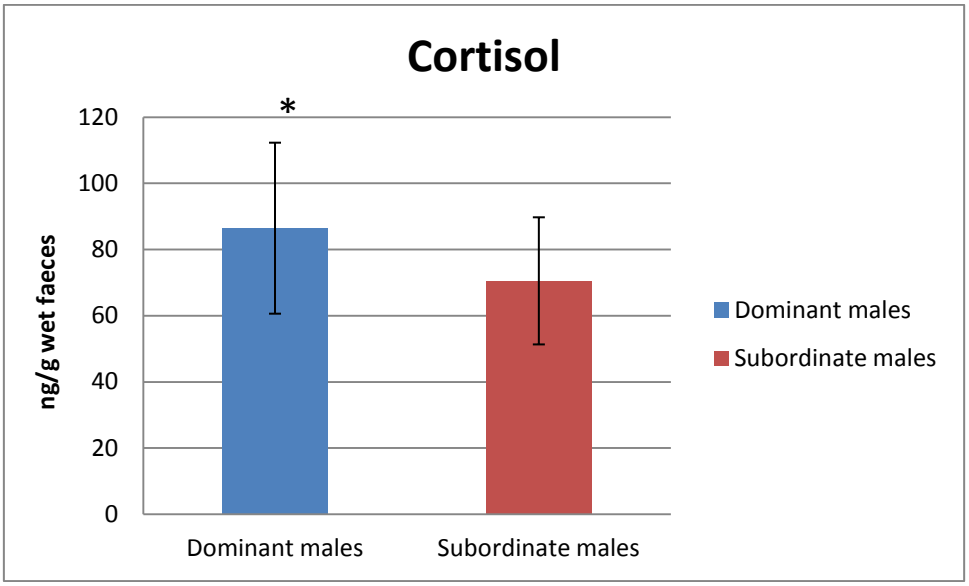

Figure 5.9: Cortisol in dominant (n=3) and subordinate (n=6) male Ethiopian wolves measured throughout the field season. The asterisk (*) denotes a significant difference. Error bars denote standard error of individual wolves.

No clear patterns in cortisol levels could be detected for individual males in Darkeena (Fig. 5.10) or Sodota/Addaa packs (Fig. 5.11). Several males show large values on single days,

such as DAR09 on day 76, DAR03 on day 92 and SOD07 on day 108, but cortisol levels do not show a consistent pattern in relation to mating or during the pup rearing time.

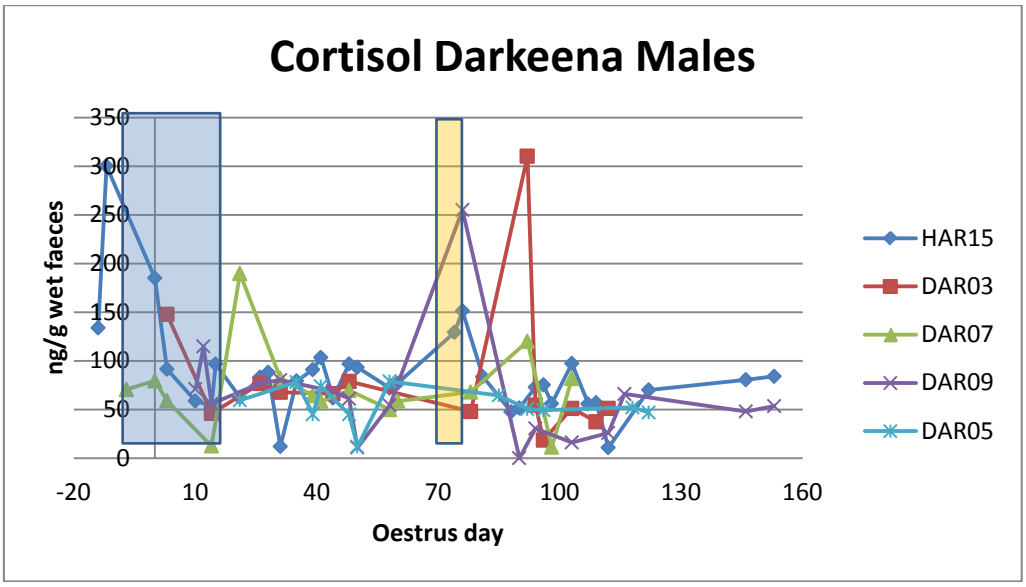

Figure 5.10: Cortisol in males in Darkeena (males listed in order of dominance). Blue shading indicates estrous (days -5 to +20) and orange shading shows the estimated time of birth. Note that DAR02 gave birth but lost her litter.

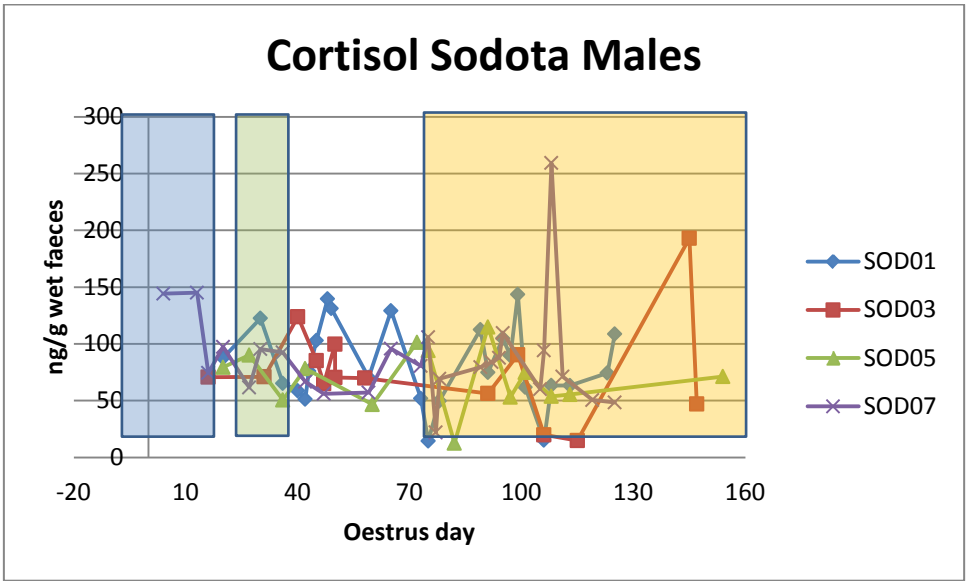

Figure 5.11: Cortisol in males in Sodota (males listed in order of dominance). Note that SOD07 is graphed together with the Sodota males as he was originally in Sodota pack. Blue shading indicates estrous (days -5 to +20) for SOD01, green shading indicates the later estrous of SOD07. Orange shading shows the time of birth/pup rearing. SOD02 gave birth and three pups emerged. SOD06 also gave birth but lost her litter.

5.4D Cortisol and Testosterone

We compared temporal patterns in testosterone and cortisol in the nine males sampled regularly in the 2007-2008 field season (Figs. 5.12, 5.13). Overall, testosterone and cortisol were significantly correlated (Pearson correlation, $p<0.005$), although patterns differed in individual males. In Sodota and Addaa packs, testosterone and cortisol were significantly correlated in Sodota's dominant male SOD01 (Pearson correlation, $p=0.003$), and Sodota subordinate males SOD03 (Pearson correlation, $p<0.005$), and SOD05 (Pearson correlation, $p=0.004$) and in Addaa dominant male SOD07 (Pearson correlation, $p=0.001$). Testosterone and cortisol were also significantly correlated in Darkeena dominant male HAR15 (Pearson correlation, $p<0.001$), and Darkeena subordinate males DAR07 (Pearson correlation, $p<0.001$), and DAR09 (Pearson correlation, $p<0.001$). Testosterone and cortisol were not correlated in Darkeena subordinate males DAR03 (Pearson correlation, $p=0.573$) and DAR05 (Pearson correlation, $p=0.105$).

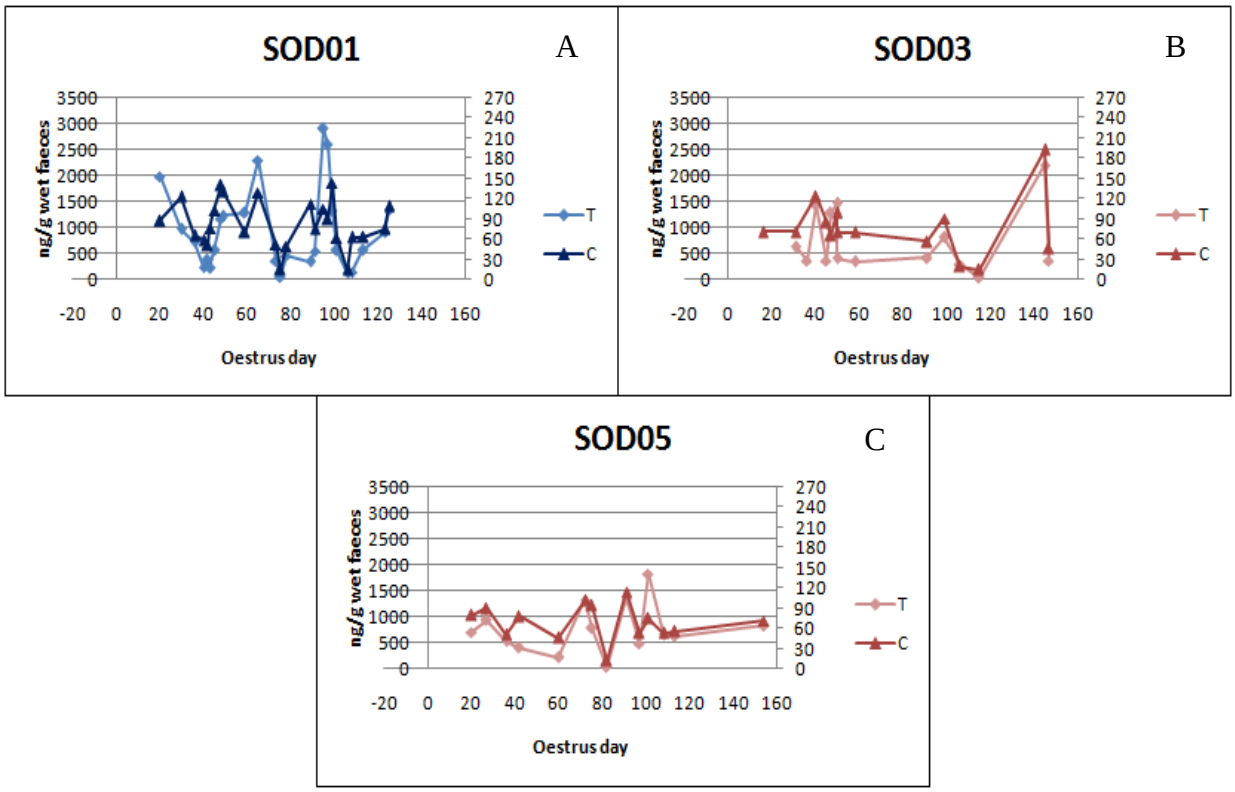

Figure 5.12: Testosterone and cortisol in Sodota dominant male SOD01 (A), and Sodota subordinate males SOD03 (B) and SOD05 (C)

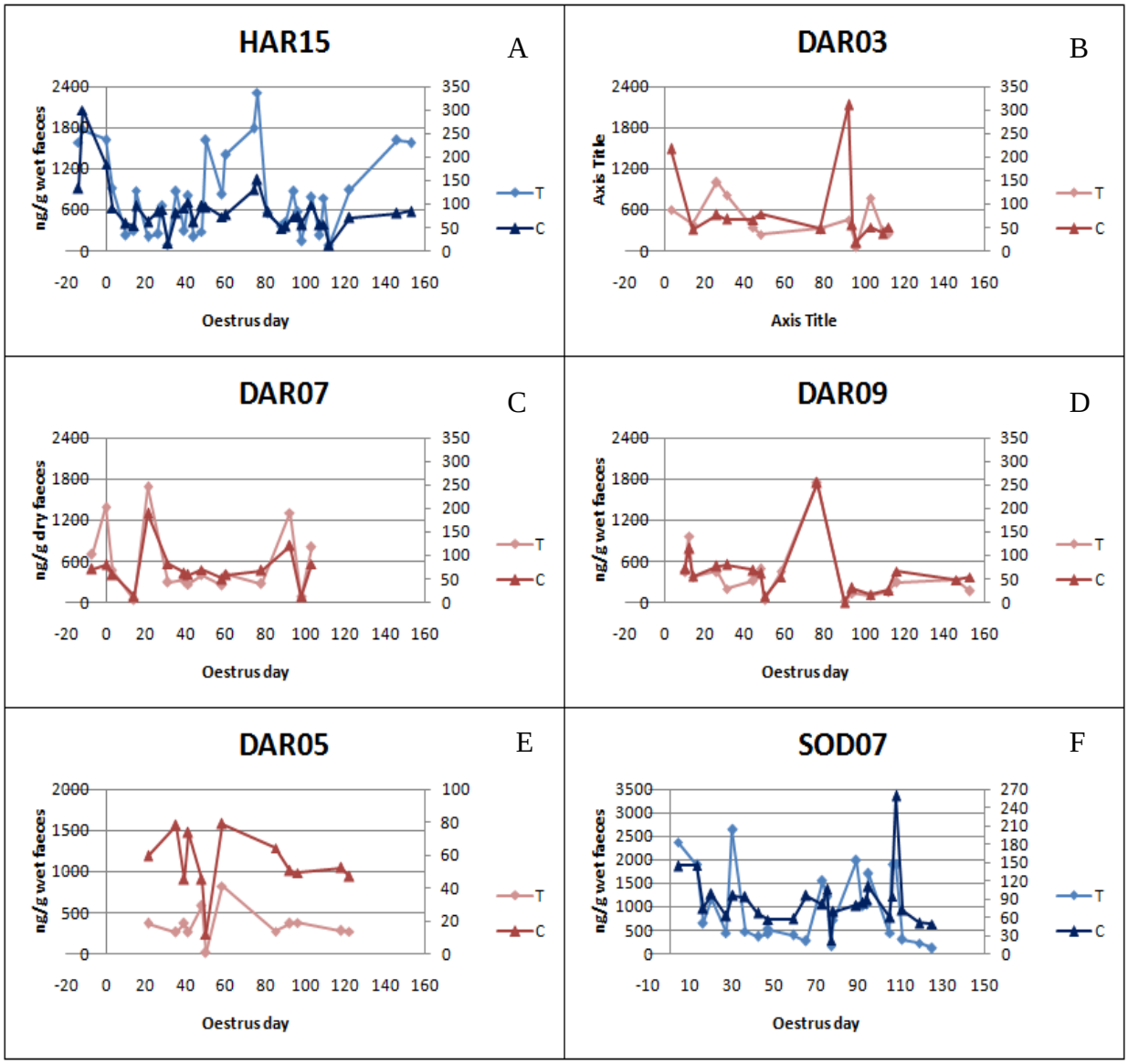

Figure 5.13: Testosterone and cortisol in Darkeena dominant male HAR15 (A), Darkeena subordinate males DAR03 (B), DAR07 (C), DAR09 (D), DAR05 (E) and Addaa dominant male SOD07 (F)

5.5 Discussion

Both dominant and subordinate males were observed engaged in mating behaviour, and on four observed occasions a subordinate male was seen trying to mate with the dominant female in the same pack. Previous work has also observed subordinates males mating (Sillero-Zubiri et al., 1996a), and genetic analysis has found multiple paternity within litters, with paternity attributed to extra-pack sires in some cases (Randall et al., 2007). These observations suggest that all males, both dominant and subordinate, display regular

reproductive behaviours and are fertile. Although data on aggressive behaviours was limited, behavioural observations suggest that dominant males are more likely to instigate both inter and intra-pack aggression, although this relationship was not statistically significant. In addition, dominant males were observed involved in aggressive behaviour relating to mate guarding. Dominant males were seen chasing subordinate males away from the dominant female with aggressive behaviours, but the reverse was never seen, i.e. subordinates, even if interested in mating with the dominant female, never tried to prevent their dominant male from mating (see also Sillero-Zubiri et al., 1996a). From the behavioural data it was difficult to determine whether there was a seasonal pattern in aggression, firstly because observations of aggression are rare, and secondly because our and EWCP's data collection efforts are not equal at all times of the year. However, previous research has shown that aggressive interactions between packs are highest during the mating season (Sillero-Zubiri & Gottelli, 1994), indicating that periods of higher aggression coincide with the mating season in Ethiopian wolves.

Frozen samples yielded testosterone levels between 12 and 2900 ng/g faeces, which is higher than the range detected in maned wolves (peaks around 1150 ng/g, Velloso et al., 1998) but lower than the range detected in African wild dogs (peaks around 75000 ng/g faeces, Creel et al., 1997a). Frozen samples yielded cortisol levels between 10.8 to 310.2 ng/g wet faeces. This is lower than values reported for male red wolves, where baseline levels were recorded to be around 354.8 ng/g faeces with peak levels around 1159.4 ng/g faeces (Young et al., 2004) but comparable to levels found in other carnivore species such as Himalayan black bear (baseline levels around 80.7 ng/g faeces with peaks around 369.6 ng/g faeces), and domestic cats (baseline levels around 213.6 ng/g faeces with peaks around 384.6 ng/g faeces, Young et al., 2004).

The results from the samples collected from Sodota, Addaa and Darkeena males in 2007-08 and those collected opportunistically from fourteen males in 2008-09 and 2009-10 show somewhat conflicting results. Although no clear seasonal patterns in testosterone levels could be detected from the males sampled in the 2007-08 field season, the opportunistically collected samples suggest that testosterone levels for dominant males were highest at oestrus, although, possbly due to low sample sizes (17 samples collected from four males), this trend was non-significant. Studies in wild populations often combine data collected from several individuals and combine these according to reproductive state (e.g. Creel et al., 1997a; Goymann et al., 2001) or in a time series (e.g. Wasser, 1996). However, as hormonal patterns can show great variation between individuals (see Chapter 4), combined data from multiple males should be interpreted with more caution than data regularly collected from known males. In addition, whereas the samples collected from males in 2007-08 were stored and transported frozen, the opportunistically collected samples were dessiccated, which gives somewhat less reliable results (see Chapter 3).

The samples collected in 2007-08 show no clear seasonal patterns for testosterone, which is somewhat surprising, given the fact that Ethiopian wolves are seasonal breeders. Although several studies in male canids have found seasonal patterns in testosterone (e.g. coyotes, Minter & DeLiberti, 2008), others have not found any seasonal patterns. For example, in captive African wild dogs, serum testosterone levels showed no seasonal effects, although testicular volume increased up to four fold in dogs in the summer as compared to spring. Spermatogenesis was also found to be seasonally dependent (Johnston et al., 2007). Similarly, Creel et al. (1997a) also found that faecal testosterone levels did not change between mating and non mating periods in wild African wild dogs, nor did testosterone levels decrease during denning. No seasonal pattern in testosterone was

detected in male maned wolves, although males were found to be aspermic outside of the breeding season (Velloso et al., 1998). These studies show that although reproductive parameters such as testicular volume and spermatogenesis may be seasonal, testosterone secretion is not necessarily seasonal. Another example comes from muriquis, *Brachyteles arachnoides*. Although muriquis are seasonal breeders, they do not show seasonal patterns in testosterone, something that is thought to be related to low levels of aggression in this species (Strier et al., 1999). Since Ethiopian wolves also generally show low levels of aggression, especially contact aggression, this may explain a lack of seasonal testosterone patterns.

Periods of mating and aggression coincide in Ethiopian wolves and male Ethiopian wolves exhibit paternal care for the dominant female's pups (Sillero-Zubiri & Gottelli, 1994). However, we found no seasonal patterns in testosterone in the nine males sampled regularly, and did not find that testosterone levels were higher during the mating season, nor lower when there where pups to care for. Based on these findings it seems that Ethiopian wolves, like dwarf mongooses (Creel et al., 1993), do not conform to the predictions of the Challenge Hypothesis (Wingfield et al., 1990). However, the samples collected opportunistically do show that dominant males (but not subordinates) have higher testosterone levels during the mating season, and lower testosterone levels when there are pups to care for. As fewer samples were collected regularly from males than from females, and males were only regularly sampled during one field season, the data are limited, especially as only three out of the nine males sampled regularly had pups to care for in the 2007-08 field season. Future studies with more regular sampling of known males should be carried out to conclusively determine patterns in testosterone secretion in dominant and subordinate male Ethiopian wolves.

Samples collected in 2007-08 (although not samples between 2008-10) show that dominant males generally have higher testosterone levels than subordinates, although, probably due to low sample sizes, this difference is not significant during oestrus or when there are pups to care for. Both Johnston et al. (2007) and Creel et al. (1997a) also found that dominant male African wild dogs had higher testosterone levels during mating times than did subordinate males.

We found higher cortisol levels in dominant males than in subordinate males, a finding that is consistent with data on African wild dogs (Creel et al., 1997a) and grey wolves (Sands & Creel, 2004). We found that dominant males were more likely to instigate both inter and intra-pack aggression, although this relationship was not statistically significant, possibly due to low samples sizes. Dominant males may also be involved in aggression relating to mate guarding. The regularly sampled dominant males also had higher testosterone levels than subordinates, and testosterone and cortisol were correlated in 7 out of 9 males sampled. Correlations between cortisol and testosterone were also found in some species such as male capuchin monkeys (Lynch et al., 2002) but not in domestic dogs (Thun et al., 1990). Although aggression was rarely recorded in this study, dominant males did show somewhat higher rates of aggression, and it is conceivable that the need for dominant males to maintain their dominant status and mate-guard their dominant female may explain why dominant males generally have higher testosterone and cortisol levels.

It is unsurprising that we did not detect a seasonal pattern in cortisol. Although reproductive hormones such as oestradiol, progesterone and testosterone often show seasonal patterns in seasonally breeding species, no clear seasonal pattern has been found

for glucocorticoids in mammals in general (Romero, 2002) or domestic dogs specifically (Thun et al., 1990). In addition, although some species including sheep, *Ovis aries* (McNatty et al., 1972), rats, *Rattus norvegicus* (D'Agostino et al., 1982) and rhesus monkeys, *Macaca mulatta* (Plant, 1981), show diurnal patterns in serum glucocorticoids, studies in domestic dogs have failed to find a convincing diurnal pattern (Kemppainen & Sartin, 1984; Kolevska et al., 2003). Since faecal samples represent a pool of hormones from several hours before defecation, diurnal effects will be much less pronounced in faecal samples than in serum samples. For this reason it is unlikely that different sampling times affected our results. However, glucocorticoid secretion is affected by adverse effects (Möstl & Palme, 2002), which may include aggression. Aggressive encounters can increase plasma concentrations of glucocorticoids (reviewed in Harding, 1981) and aggressive behaviour in itself can be stressful (e.g. Rosado et al., 2010). Dominant males showed somewhat higher rates of aggression than subordinates, and they may have to act aggressively more often to maintain their dominant status (Creel, 2001). This may explain why dominant males had higher average cortisol levels than subordinates.

The behavioural observations, together with the physiological data, as well as previous research, suggest that subordinate males are behaviourally, but not hormonally reproductively suppressed. Subordinate males do exhibit mating behaviour (see also Sillero-Zubiri et al., 1996a), and have been known to sire pups (Randall et al., 2007). However, subordinate males seem to be prevented from mating with their own dominant female by the dominant male. Since male Ethiopian wolves are generally philopatric (Sillero-Zubiri et al., 1996a), dominant and subordinate males are often related. If subordinate males succeed in mating with neighbouring females and sire pups with them, this will provide inclusive fitness for the pack's dominant male. As subordinate males do

not invest paternal care in their extra-pack offspring, but do invest paternal care in their own pack's offspring, subordinate males siring extra-pack pups has no detrimental effect for the pack's dominant male, or his pups with the dominant female. This inclusive fitness may be explain why subordinate males are not hormonally reproductively suppressed.

Chapter 6: General Discussion

6.1 Reproductive physiology of Ethiopian wolves

In this thesis I aimed to understand the reproductive physiology of male and female Ethiopian wolves, and especially the reproductive suppression of subordinate wolves. The collection of faeces, and extraction and assaying of faecal hormones enables researchers to study reproductive physiology and social stress non-invasively, without capturing or handling focal animals (Buchanan & Goldsmith, 2004). This is especially useful when studying wild populations in which frequent handling of animals is not practical or justifiable, such as the one studied here.

This study took place in Ethiopia's Bale Mountains National Park, which is home to more than half of the remaining Ethiopian wolf population (Sillero-Zubiri & Gottelli, 1994). Ethiopian wolves are cooperative breeders, and within a pack only the dominant pair breeds. However, 70% of 30 mating events observed by Sillero-Zubiri et al. (1996a) involved a dominant female and males (dominant or subordinate) from neighbouring packs. All pack members help rear the pups, which includes den guarding and regurgitating prey to pups, and subordinate females may allosuckle the dominant female's pups (Sillero-Zubiri & Gottelli, 1994). Ethiopian wolves are seasonal breeders, with one breeding season per year and pups are born towards the end of the rainy season (Sillero-Zubiri et al., 1998). Although previous studies have given us some insight into Ethiopian wolf reproduction using behavioural observations and molecular genetics, their reproductive physiology has not been previously studied.

Based on previous research in other canid species (Chapter 2), I was able to ask questions and make predictions about Ethiopian wolf reproductive physiology. Since Ethiopian wolves are seasonal breeders, we expected to see seasonal patterns in reproductive

hormones such as oestradiol, progesterone and testosterone. Seasonal trends in oestradiol and progesterone have been found in other canids such as female African wild dogs (Creel et al., 1997a) and grey wolves (Seal et al., 1979), and male coyotes (Minter & DeLiberti, 2008) and red wolves (Walker et al., 2002) show seasonal trends in testosterone, with highest levels during the breeding season. Although Ethiopian wolves generally show low levels of aggression (Sillero-Zubiri, 1994), higher cortisol levels have been reported in dominants in wild populations of other communally breeding canids, which may be related to the cost of maintaining dominance status (Creel, 2001). This body of research (see Chapters 1 and 2) provided the necessary background knowledge against which I investigated specific questions relating to female and male Ethiopian wolf reproductive physiology.

6.2 Considerations for faecal hormone studies in wild populations

In recent years studies of reproductive physiology and/or social stress in wild populations have been increasingly carried out by assaying hormones extracted from faecal or urine samples (e.g. Brockman & Whitten, 1996; Moss et al., 2001; Cavigelli, 1999; Garcia Pereira et al., 2005). As collecting faecal or urine samples is non-invasive, it provides an ideal method of studying wild animals that cannot or should not be regularly trapped and sampled invasively, such as Ethiopian wolves, which exist only in the wild (Sillero-Zubiri & Macdonald, 1997). However, despite the many advantages of this method, there are also several disadvantages when compared with studies done in captivity, and these should be taken into consideration. Studies in captivity allow for very controlled circumstances, such as regular sampling (either serum or faecal/urine samples), regular behavioural observations, consistent housing and social conditions, and even a consistent diet, as well as allowing for extra measurements that cannot be done in the wild such as observations on

vaginal cytology (e.g. Carlson & Gese, 2008), or testes size (e.g. Johnston et al., 2007). Samples collected from captive populations can also easily be stored frozen, which is the preferred method of storing faecal samples (e.g. Terio et al., 2002).

In the wild, conditions are much more variable, which may affect results. For instance, stressful situations can be controlled and minimized in captivity, but wild populations are subject to stressors that cannot be controlled, or even observed by researchers. Similarly, imposing a regular sampling schedule is often impractical or even impossible in wild populations. Storing the samples collected from wild populations is also much more challenging. Other factors that potentially affect hormones measured in faecal samples may include diet (Whitten et al., 1998), although this effect has not been well studied. Although Ethiopian wolves specialize in hunting rodents (Sillero-Zubiri & Gottelli, 1995a), we found that the consistency of faeces may vary. Although any observed undigested parts such as bones were always removed from faecal samples prior to collection and preservation, faeces may consist of liquid diarrhoea, and on several occasions wolves were observed ingesting vegetation and excreting slimy, grassy faeces. Some faeces also contained blood. These different faecal consistencies may have affected hormone levels measured in the samples collected.

Since no Ethiopian wolves have ever been kept or bred in captivity (Sillero-Zubiri & Macdonald, 1997), the reproductive physiology of this species could only be studied non-invasively in a wild population. The potential pitfalls of studying reproductive physiology and social stress in a wild population of Ethiopian wolves were recognized, and I tried to mitigate these as much as possible. One important step to limit errors was to validate a method of storing Ethiopian wolf faecal samples (Chapter 3), as recommended by several

authors (Buchanan & Goldsmith, 2004; Terio et al., 2002). Similarly, I validated the extraction protocol used for the samples (section 1.10), and validated the protocols used to measure progesterone, oestradiol, cortisol and testosterone using inter and intra-assay controls and parallelism studies (section 1.13) as recommended by several authors (e.g. Buchanan & Goldsmith, 2004). In the field, efforts were made to sample individuals regularly by going to each pack territory on fixed days each week. However, it was not always possible to sample focal animals regularly due to climatological factors such as thick fog or simply due to failure to find a specific wolf, or not observing a focal animal defecating on a certain day. In addition, it was impossible to always collect faecal samples at the same time of day. These issues lead to an irregular sampling frequency, which complicated the interpretation of the data. This study was also negatively affected by the outbreak of a rabies epizootic in the Web Valley in 2008-2009, which negatively impacted the sample size due to a ~76% mortality in focal packs (Johnson et al., 2010). Despite the challenges associated with this study, 23 adult females and 9 adult males were regularly sampled, and 14 males were opportunistically sampled over the course of this study, with a total of 1,085 faecal samples collected and analyzed in order to assess Ethiopian wolf reproductive physiology non-invasively.

6.3 Preservation of Ethiopian wolf faecal samples for hormone analysis

Although hormone assay technologies were originally applied to domesticated and captive species, non-invasive studies of reproductive physiology and/or social stress are increasingly being carried out in wild populations (for a review see Schwarzenberger, 2007). Freezing samples is considered the preferred method of preserving faecal samples for hormone analysis (e.g. Terio et al., 2002), but this often impractical when studying wild populations. Freezing Ethiopian wolf faecal samples collected in Bale Mountains National

Park was difficult due to logistical problems, and a practical alternative method of preserving Ethiopian wolf faecal samples was needed. By comparing concentrations of oestradiol, progesterone, testosterone and cortisol in methanol extracted and desiccated Ethiopian wolf faecal samples to frozen (control) samples, we found that desiccation provided a practical and reliable way of preserving the faecal samples for hormone analysis, without the need for freezing, and used this preservation method for the remainder of this three year study.

6.4 Oestradiol, progesterone and cortisol in female Ethiopian wolves

A total of 819 samples from female Ethiopian wolves were assayed for oestradiol, progesterone and cortisol. In dominant females, I expected to see only one main oestradiol surge in the year, coinciding with the mating season. Since subordinate females rarely mate (Sillero-Zubiri et al., 1996a) or have pups (Randall et al., 2007), I predicted that most subordinate females would not come into oestrus at the same time as their dominant females, and as such, would not show an oestradiol peak. These predictions were largely substantiated. In 13 out of 14 dominant females, I did detect an oestradiol peak during the mating season (defined as an increase of at least 2.6 fold over baseline levels). In one female (SOD02) I failed to detect an oestradiol peak; this may have been due to an insufficient sampling frequency around oestrus. Oestrus was further confirmed in all dominant females through observations of mating behaviour, pregnancy and birth of pups or, in one case, a post mortem examination. In contrast, none of the nine subordinate females showed an oestradiol peak, or oestrus behaviour during the annual mating season. However, two subordinate females (DAR12 and BBC42) and one previously subordinate female (SOD06) did show oestradiol peaks and oestrus (mating) behaviour outside of the annual mating season. Two other subordinate females (DAR10 and DUM04) showed

oestradiol peaks outside of the annual mating season, although no oestrus behaviour was observed. When all these data were combined, oestradiol levels in dominant females were found to be significantly higher than those of subordinate females during the annual mating season, implying hormonal reproductive suppression in subordinate females. Although anecdotal, the case of MEG06 further supports this conclusion. When MEG06 was subordinate to MEG02, she showed no oestradiol peaks during the mating season, whereas MEG02 did show a large oestradiol peak, mated, became pregnant and successfully reared one pup. A year later, when MEG02 had dispersed and MEG06 became the dominant (and only) female in the pack, MEG06 did come into oestrus, showed a large oestradiol peak and mated and bred successfully. These data provide strong evidence for a hormonal mechanism of reproductive suppression in subordinate female Ethiopian wolves.

Ten out of thirteen females who were confirmed to be pregnant showed higher progesterone levels during pregnancy, although in some cases (e.g. SOD06 in 2007), this increase was limited to very early pregnancy. In three females I failed to detect a clear increase in progesterone, although pregnancy was reliably established through observed mating and/or birth of pups. In all three of these cases, progesterone increased above baseline values as early as 60 days before estimated conception (SOD02 in 2009). These patterns are not easily explained, but may be indicative of split oestrus (Meyers-Wallen, 2007). Seven out of nine subordinate females also showed an increase in progesterone whilst their dominant females were pregnant, and three also showed physical signs such as extended abdomen or allosuckling of pups, although they were never seen mating and showed no signs of having given birth. These results suggest that female Ethiopian wolves may become pseudopregnant, something which is relatively common in canid species (Asa & Valdespino, 1998; Concannon et al., 2009). Pseudopregnancy in canids is the result of

an infertile ovulation (Concannon, et al., 2009), and is thought to be adaptive and prepare subordinate females for their role as 'helpers' for their dominant female's pups (Macdonald, 1980). While no subordinate females showed signs of oestrus or oestradiol peaks during the annual mating season, several subordinate females did show signs of oestrus outside of the mating season. It is possible that some subordinate females ovulated outside of the annual mating season, and that this would have enabled them to become pseudopregnant.

Contrary to expectations, there were no significant differences in cortisol levels between dominant and subordinate females, indicating that subordinate females were not mainly reproductively suppressed through increased cortisol levels. Subordinate females did however have non-significantly higher average cortisol levels, which may be a result of subordinate females being on the receiving end of intra-pack aggression much more often than dominant females. In species in which subordinates are more likely to be the target of aggressive behaviours by higher ranking individuals, subordinates may have higher cortisol than do dominants (e.g. baboons, *Papio cynocephalus*, Sapolsky et al., 1997). Nevertheless, rather than subordinate females having consistently higher cortisol levels, several females showed high cortisol spikes at different times of the breeding season which probably corresponded with unobserved stressful events.

There are at least three possible alternative explanations for potentially stressful circumstances affecting the Ethiopian wolves included in this study. Firstly, the females assayed for cortisol all lived in the Web Valley, which is the area in the Bale Mountains National Park most densely populated by Oromo pastoralists. On occasion, local people may harass wolves by chasing them away or throwing rocks at them (Sillero-Zubiri 1994;

personal observation), and events like these may cause short-term increases in cortisol. Secondly, it is not uncommon for domestic dogs in the Web Valley to interact with and behave aggressively towards Ethiopian wolves (Sillero-Zubiri 1994; personal observation), which may also stress them. Lastly, many of the samples assayed for cortisol were collected during a period when the wolf population in the Web Valley was affected by the 2008-09 rabies epizootic (Johnson et al., 2010), and this would most likely have affected cortisol levels.

Subordinate female Ethiopian wolves can choose between several reproductive strategies (Sillero-Zubiri et al., 1996a). They may choose to stay in their natal pack, help rear related pups, and have the chance of one day becoming the dominant, breeding female in their natal pack; they may disperse and seek a breeding opportunity in a different pack (Sillero-Zubiri et al., 1996a); or they may try to split an existing pack (pack fission) and become the dominant female in the new pack (Marino, 2003a). Sillero-Zubiri et al. (1996a) found that achieving dominant status was the only way for females to breed successfully and that resident females had the advantage over floaters in a competition for a breeding position. Furthermore, five out of ten dominant females included in Sillero-Zubiri et al.'s (1996) study kept their dominant position for the duration of the four year study, and dominant females were replaced only after their deaths. Therefore, the strategy of staying in a natal pack provided females with a higher likelihood of achieving dominant status and, once they achieved this status, they were likely to maintain it until their death, thus possibly having several years in which to breed. However, in packs with multiple females, subordinates may have a better chance of breeding if they disperse, particularly when there are older subordinate females 'in line' to inherit the dominant position ahead of them. Pack fission may be a good strategy for subordinate females, especially since in pack fission

subordinate females were often joined by subordinate males from their natal pack, and may therefore more easily carve out a new territory (Marino et al., submitted, C. Sillero-Zubiri, pers. comm.). Successful pack fissions have been recorded, for example in 1999 when five wolves split from Mulamo pack to form Darkeena pack (Marino, 2003a) and when SOD06 and SOD07 split from Sodota pack to form Addaa pack (this study, Chapter 4). However, attempted pack fissions often fail, possibly due to aggression from the natal pack's dominant female (Sillero-Zubiri et al., 1996a). Failed pack fission attempts include Sodota pack in 1990 (Sillero-Zubiri et al., 1996a) and BBC pack in 1999 (Marino, 2003a). During this three year study only one new pack was established through fission (Addaa pack), and one other subordinate female (MEG06) achieved dominant status after the rabies epizootic, when her dominant female dispersed.

It may be that there is a physiological basis to subordinate females' decisions regarding breeding strategies. However, this is difficult to ascertain, especially as sample sizes were limited (i.e. only one successful pack fission, and only one other change from subordinate to dominant status in this study). However, on the basis of my findings it is possible to speculate on the role of reproductive physiology in female choice.

Subordinate females were found to have higher average cortisol levels than dominant females (although this effect was non-significant), and subordinates were often the target of intra-pack aggression. Subordinate females received aggression not only from the dominant female, but also from higher ranking subordinate females and from males. This aggression and increased stress may play a role in females' decisions to stay or to disperse, and Sillero-Zubiri et al. (1996a) observed that harassment by dominant females or higher ranking females could lead to expulsion and dispersal of subordinate females.

The one case of pack fission recorded in this study involved SOD06. Interestingly, SOD06 was also the only subordinate female in the study who showed an oestradiol peak at the same time as her dominant female, SOD02. SOD06 then split from Sodota pack with a male (SOD07), and was seen mating a month after the annual mating season, when she again showed an oestradiol peak. A splitting attempt recorded in 1999 in Sodota pack also involved a subordinate attempting to split from her natal pack and this female had pups, although they did not survive. There were strong suspicions that the Sodota dominant female had killed these pups, since she was observed in the vicinity of, and entering, the subordinate's den (Sillero-Zubiri et al., 1996a). It may be that in some cases dominant females fail to reproductively suppress their subordinate females during the annual mating season, who then come into oestrus. This may be a determining factor in a subordinate's decision to split from her natal packs and attempt to breed. In summary, it seems that subordinates may be willing to tolerate reproductive suppression since their best chance of breeding is to eventually become the dominant, breeding female in their natal pack, but that harassment, stress and occasional failures of reproductive suppression may play a role in subordinate female's decisions to disperse or split from their natal pack.

6.5 Testosterone and cortisol in male Ethiopian wolves

Despite the fact that Ethiopian wolves are seasonal breeders, I could not detect clear patterns of testosterone levels in the nine males sampled regularly, although opportunistically collected samples did suggest that dominant males had higher testosterone levels during the mating season. Regularly sampled males did not show seasonal testosterone patterns either as individuals, nor when data were combined according to dominance rank and time of year (oestrus, non-oestrus and presence of pups). However, as two focal females lost their pups in 2007, data from males showing paternal

care was limited to three males in Sodota pack. Ideally, more males would be regularly sampled to further elucidate if testosterone levels are lower in Ethiopian wolves when they are showing paternal care to the pack's pups. Regularly sampled dominant males had higher testosterone levels than did subordinates, although both dominant and subordinate males were observed engaging in mating behaviour, and we know from previous studies that both dominant and subordinate males may mate and sire pups (Randall et al., 2007; Sillero-Zubiri et al., 1996a). As expected, we did not find seasonal patterns in cortisol either, and, although aggressive interactions are more common during the annual mating season (Sillero-Zubiri et al., 1994, Sillero-Zubiri et al., 1998), cortisol levels were not significantly higher during the mating season in either dominant or subordinate males. Dominant males, did however, have higher average cortisol levels that did subordinates. The need for the dominant male to maintain his status and guard his female may explain the higher testosterone and cortisol levels found.

Whereas female Ethiopian wolves are more likely to disperse, males are usually philopatric and stay in their natal pack (Sillero-Zubiri et al., 1996a). This means that the dominant and subordinate males in a pack are often closely related. Although the dominant male prevents subordinates from mating with the dominant female in the same pack (Sillero-Zubiri et al., 1996a, Chapter 5, this study), subordinate males do not appear to be hormonally reproductively suppressed, as evidenced by observations of subordinates mating (Sillero-Zubiri et al., 1996a, Chapter 5, this study), and extra-pack paternity in litters (Randall et al., 2007). All pack members, including subordinate males, help rear the dominant pair's pups (Sillero-Zubiri et al., 1996), and subordinate males, even if they have mated with females in other packs, remain in their natal packs. It is possible that extra-pack copulations by subordinate males provide dominant males with a degree of inclusive

fitness, as subordinate and dominant males are likely to be closely related, and this may explain why subordinate males are behaviourally, but not hormonally, reproductively suppressed.

6.6 Reproductive physiology and conservation of Ethiopian wolves

A series of measures have been proposed for the conservation of Ethiopian wolves, including reproductive management such as egg cell and semen banking and captive breeding (Sillero-Zubiri et al., 2004a; Sillero-Zubiri & Macdonald, 1997). Captive breeding can be used as a conservation tool and ideally, captive populations would be used to re-establish wild populations (e.g. Kleiman, 1989). Several examples of successful captive breeding programmes and/or reintroductions of carnivore species exist, the best known of which is possibly the black footed ferret, *Mustela nigripes* (e.g. May, 1986). Black footed ferrets were thought to be extinct in the wild due to a combination of extermination of their main prey (prairie dogs, *Cynomys leucurus*, May, 1986) and mortality from canine distemper (Williams et al., 1988). However, a surviving population was discovered in Wyoming in 1981 (May, 1986). A captive breeding population was subsequently established and black footed ferrets were successfully reintroduced into the wild (Dobson & Lyles, 2000). Captive populations of Mexican wolves, *Canis lupus baileyi*, and red wolves, *Canis rufus*, have also been successfully established, and both species have been reintroduced into the wild, with mixed success (Hedrick & Fredickson, 2008). Similarly, a captive population of African wild dogs was established in 1954, and the African wild dog captive breeding programme is considered a success (Frantzen et al., 2001). African wild dogs have since been re-introduced to several areas (Gusset et al., 2008). Captive breeding and reproductive management have their limitations (Snyder et

al., 1996), and should not be seen as substitutes for in-situ conservation. Nevertheless, these measures can be used as a 'last resort' for species that face extinction in the wild.

More than half of the roughly 500 remaining Ethiopian wolves are found in the Bale Mountains National Park, with small populations in other high altitude areas in Ethiopia (Marino, 2003b). The species' is under threat from human encroachment and habitat loss. Even inside Bale Mountains National Park, human settlement and cattle grazing, as well as conversion of land for agriculture, have been on the increase since the Park was established (Stephens et al., 2001). However, arguably the most immediate threat to Ethiopian wolves is disease, with outbreaks of rabies, but also canine distemper affecting Ethiopian wolves in Bale Mountains National Park (e.g. Johnson et al., 2010; Laurenson et al., 1998; Randall et al., 2004). Rabies and canine distemper are thought to be transmitted to wolves through the domestic dogs that accompany people and their livestock living in Ethiopian wolf habitat (Laurenson et al., 1998). Rabies greatly reduced Ethiopian wolf numbers in Bale in 1992 (Sillero-Zubiri et al., 1996b), 2003 (Haydon et al., 2006), and most recently in 2008-2009 (Johnson et al., 2010), and canine distemper was suspected to have infected Ethiopian wolves in 1993 (Laurenson et al., 1998) and in 2010 (C. Gordon, pers. comm.). The most recent rabies epizootic, which affected the study population subject of this thesis, highlights once again the vulnerability of this endangered canid. Between August 2007 and January 2009, ~76% of all the Web Valley wolves in the focal packs in this study died or disappeared in the rabies epizootic (Johnson et al., 2010, personal observation). Although the disease then spread South to the nearby Morebawa population, further spread of rabies was prevented by an emergency vaccination intervention (Stewart et al., 2010). In 2010 an outbreak of canine distemper was discovered in Bale, and the final mortality rate from this latest epizootic is yet to be established (C. Gordon, pers. comm.).

The fact that even the largest population of this species, living inside a legally protected national park, is susceptible to such devastating disease outbreaks emphasizes how vulnerable the remaining populations of Ethiopian wolves are to local extirpation, and how important further conservation initiatives are for this species. Although protection of their natural habitat is the best conservation approach for Ethiopian wolves, this is problematic due to the fact that Bale Mountains National Park, as well as other Afroalpine regions are quite densely populated by people who depend on these areas for their livelihoods (Stephens et al., 2001). Although several organizations including the Ethiopian Wildlife Conservation Authority, the Oromia Bureau of Agriculture and Rural Development and the Frankfurt Zoological Society are working towards sustainable management of the Park, the reality is that this may take many years to implement. In the meantime, other conservation measures, including reproductive management of Ethiopian wolves should be considered. Given the immediate problems surrounding the conservation of Ethiopian wolf habitat and the threats this species faces, I believe these measures could make a useful contribution to the long term conservation of this rare canid.

As outlined by Sillero-Zubiri and Macdonald (1997), conservation measures for Ethiopian wolves should include semen and egg cell banking, metapopulation management and captive breeding. Sound reproductive management should always be based on knowledge and understanding of a species' reproduction. This realization developed among reproductive biologist after several species, most notably cheetahs, failed to reproduce in captivity using assisted reproductive technologies developed in domesticated species such as cows (Wildt et al., 2001). Instead, the reproductive physiology of each new species should be studied, and this knowledge should inform reproductive management if it is to

be successful (Wildt et al., 2001). Some aspects of Ethiopian wolf reproduction have been previously studied using behavioural and molecular tools (Gottelli et al., 1994; Randall et al., 2007; Sillero-Zubiri, 1994; Sillero-Zubiri et al., 1996a; Sillero-Zubiri et al., 1998), and this study provides new insights into the reproductive physiology of this species. This knowledge should be applied to reproductive management of Ethiopian wolves, and some reproductive initiatives are being developed.

Plans are currently underway for electro-ejaculation and semen harvesting trials in male Ethiopian wolves, and future meta-population management plans may include assisted reproduction and the establishment of a captive breeding population (Sillero-Zubiri et al., 2004a; Sillero-Zubiri & Macdonald, 1997). This study has provided, for the first time, a more detailed understanding of the reproductive physiology of Ethiopian wolves, and the knowledge gained here should be used to inform future reproductive management initiatives. For instance, as my results indicate that subordinate females did not usually ovulate during the annual mating season, only dominant females should be selected for egg cell harvesting. Similarly, my results show that subordinate females are hormonally reproductively suppressed in the presence of a dominant female, but that they may come into oestrus and breed when removed from their dominant female. This should be considered in any eventual captive breeding. To maximize captive breeding success, females should not be housed together, as dominants are likely to reproductively suppress subordinates. If a captive breeding population were to be established, it would be possible to carry out further studies on reproductive physiology on captive wolves, including on vaginal cytology in females and seasonal trends in testicle volume and spermatogenesis in males. We did not find clear seasonal patterns in testosterone secretion. However, other canids such as maned wolves (Velloso, et al., 1998) and African wild dogs (Johnston et al.,

2007) did not show seasonal patterns in testosterone either, despite showing seasonal patterns in testicle size and spermatogenesis. Maned wolves were found to be aspermic outside of the breeding season (Velloso, et al., 1998). For this reason, despite not finding seasonal trends in testosterone levels, I would recommend that any collection of Ethiopian wolf semen take place during the annual mating season. The results of this thesis represent a contribution to the current state of knowledge of this species' biology and behaviour, and will help inform future reproductive management of Ethiopian wolves, and in doing so, contribute to the conservation of this charismatic endangered canid.

References

Andersen, B. K., Wiger, R., Dahl, E., Torp, T., Farstad, W., Krogenaes, A., McNeilly, A. S., Paulenz, H. & Ropstad, E. 2001. Seasonal changes in spermatogenic activity and in plasma levels of FSH, LH and testosterone and the effect of immunization against inhibin in the male silver fox (*Vulpes vulpes*). *International Journal of Andrology*, **24,** 284-294.

Arnold, W. & Dittami, J. 1997. Reproductive suppression in male alpine marmots. *Animal Behaviour*, **53,** 53-66.

Asa, C. S., Bauman, J. E., Coonan, T. J. & Gray, M. M. 2007. Evidence for induced estrus or ovulation in a canid, the island fox (*Urocyon littoralis*). *Journal of Mammalogy*, **88,** 436-440.

Asa, C. S. & Valdespino, C. 1998. Canid reproductive biology: an integration of proximate mechanisms and ultimate causes. *American Zoologist*, **38,** 251-259.

Asa, C. S., Valdespino, C. & Cuzin, F. 2004. Fennec fox, *Vulpes zerda*. In: *Canids: foxes, wolves, jackals and dogs. Status Survey and Conservation Action Plan* (Ed. by Sillero-Zubiri, C., Hoffmann, M. & Macdonald, D. W.). Gland, Swizerland ans Cambridge, UK: IUCN/SSC Canid Specialist Group.

Asikainen, J., Mustonen, A., Hyvärinen, H. & Nieminen, P. 2003. Seasonal reproductive endocrine profile of the raccoon dog (*Nyctereutes procyonoides*) - effects of melatonin and food deprivation. *Journal of Experimental Zoology*, **299A,** 180-187.

Balshine-Earn, S., Neat, F. C., Reid, H. & Taborsky, M. 1998. Paying to stay or paying to breed? Field evidence for direct benefits of helping behavior in cooperatively breeding fish. *Behavioral Ecology*, **5,** 532-538.

Bamberg, E. & Schwarzenberger, F. 1990. Fecal steroid assay for monitoring of estrous cycle and pregnancy. In: *IVth Congress of the International Society for Animal Clinical Biochemistry* (Ed. by Kaneko, J. J.). University of California: Davis.

Barrett, G. M., Shimizu, K., Bardi, M., Asaba, S. & Mori, A. 2002. Endocrine correlates of rank, reproduction, and female-directed aggression in male Japanese macaques (*Macaca fuscata*). *Hormones and Behavior*, **42,** 85-96.

Barrett, J., Abbott, D. H. & George, L. M. 1990. Extension of reproductive suppression by pheromonal cues in subordinate female marmoset monkeys, *Callithrix jacchus*. *Journal of Reproduction and Fertility*, **90,** 411-418.

Beach, F. A., Dunbar, I. F. & Buehler, M. G. 1982. Sexual characteristics of female dogs during successive phases of the ovarian cycle. *Hormones and Behavior*, **16,** 414-442.

Becker, J. B. & Breeddlove, S. M. 2002. Introduction to behavioral endocrinology. In: *Behavioral Endocrinology* (Ed. by Becker, J. B. & Breeddlove, S. M.). Cambridge, Massachusetts: Massachusetts Institute of Technology.

Beehner, J. C. & Whitten, P. L. 2004. Modifications of a field method for fecal steroid analysis in baboons. *Physiology and Behavior*, **82,** 269-277.

Bennett, N. C. 2009. Reproductive suppression in social *Cryptomys damarensis* colonies - a lifetime of socially-induced sterility in males and females (Rodentia: Bathyergidae). *Journal of Zoology*, **234,** 25-39.

Bennett, N. C., Faulkes, C. G. & Molteno, A. T. 1996. Reproductive suppression in subordinate, non-breeding female Damaraland mole-rats: two components to a lifetime of induced infertility. *Proceedings of the Royal Society: Biological Sciences*, **263,** 1599-1603.

Blanchard, R. J., McKittrick, C. R. & Blanchard, D. c. 2001. Animal models of social stress: effects on behavior and brain neurochemical systems. *Physiology and Behavior*, **73,** 261-271.

Bonnin, M., Mondain-Monval, M. & Dutourne, B. 1978. Oestrogen and progesterone concentrations in peripheral blood in pregnant red foxes (*Vulpes vulpes*) *Journal of Reproduction and Fertility*, **54,** 37-41.

Bouchard, S., Youngquist, R.S, Vaillancourt, D, Krause, G.F, Guay, P, Paradis, M. 1991. Seasonality and variability of the interestrous interval in the bitch. *Theriogenology*, **36,** 41-50.

Boue, F., Delhomme, A. & Chaffaux, S. 2000. Reproductive management of silver foxes (*Vulpes vulpes*) in captivity. *Theriogenology*, **53,** 1717-1728.

Boutelle, S. M. & Bertschinger, H. J. 2010. Reproductive management in captive and wild canids: contraception challenges. *International Zoo Yearbook*, **44,** 109-120.

Braastad, B. O. & Bakken, M. 1993. Maternal infanticide and periparturient behaviour in farmed silver foxes *Vulpes vulpes*. *Applied Animal Behaviour Science*, **36,** 347-361.

Brockman, D. K. & Whitten, P. L. 1996. Reproduction in free-ranging *Propithecus verreauxi*: estrus and the relationship between multiple partner matings and fertilization. *American Journal of Physical Anthropology*, **100,** 57-69.

Brown, J. L. 1978. Avian communal breeding systems. *Annual Review of Ecological Systems*, **9,** 123-155.

Brown, J. L., Terio, K. A. & Graham, L. H. 1996. Fecal androgen metabolite analysis for non invasive monitoring of testicular steroidogenic activity in felids. *Zoo Biology*, **15,** 425-434.

Brown, J. L., Wasser, S. K., Wildt, D. E. & Graham, L. H. 1994. Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured noninvasively in feces. *Biology of Reproduction*, **51,** 776-786.

Buchanan, K. L. & Goldsmith, A. R. 2004. Noninvasive endocrine data for behavioural studies: the importance of validation. *Animal Behaviour*, **67,** 183-185.

Burke, A. 1996. Wattled crane, *Bugeranus caruncalutus* In: *The cranes: status survey and conservation action plan* (Ed. by Meine, C. & Archibald, G.). Gland, Switzerland and Cambridge, UK: IUCN.

Carlson, A. A. & Gese, E. M. 2007. Relaxin as a diagnostic tool for pregnancy in the coyote, *Canis latrans*. *Animal Reproduction Science*, **101,** 304-312.

Carlson, D. A. & Gese, E. M. 2008. Reproductive biology of the coyote (*Canis latrans*): integration of mating behavior, reproductive hormones, and vaginal cytology. *Journal of Mammalogy*, **89,** 654-664.

Cavigelli, S. A. 1999. Behavioural patterns associated with faecal cortisol levels in free-ranging female ring-tailed lemurs, *Lemur catta*. *Animal Behaviour*, **57,** 935-944.

Cavigelli, S. A., Dubovick, T., Levash, W., Jolly, A. & Pitts, A. 2003. Female dominance status and fecal corticoids in a cooperative breeder with low reproductive skew: ring-tailed lemurs (*Lemur catta*). *Hormones and Behavior*, **43,** 166-179.

Cavigelli, S. A. & Pereira, M. E. 2000. Mating season aggression and fecal testosterone levels in male ring tailed lemurs (*Lemur catta*). *Hormones and Behavior*, **37,** 246-255.

Chakraborty, P. K. 1987. Reproductive hormone concentrations during estrus, pregnancy, and pseudopregnancy in the labrador bitch. *Theriogenology*, **27,** 827-840.

Chapeau, C., King, G. J. & Bamberg, E. 1993. Fecal estrogens in one primate and several ungulate species during various reproductive stages. *Animal Reproduction Science*, **34,** 167-175.

Chen, H., Chandrashekar, V. & Zirkin, B. R. 1994. Can spermatogenesis be maintained quantitatively in intact adult rats with exogenously administered dihydrotestosterone? *Journal of Andrology*, **15,** 132-138.

Chrousos, G. P. & Gold, P. W. 1992. The concepts of stress and stress system disorders. *Journal of the American Medical Association*, **269,** 1244-1253.

Cockburn, A. 1998. Evolution of helping behaviour in cooperatively breeding birds. *Annual Review of Ecological Systems*, **29,** 141-177.

Concannon, P. W., Butler, P. W., Hansel, W., Knight, P. J. & Hamilton, J. M. 1978. Parturition and lactation in the bitch: serum progesterone, cortisol, and prolactin. *Biology of Reproduction*, **19,** 1113-1118.

Concannon, P. W., Castracane, V. D., Temple, M. & Montanez, A. 2009. Endocrine control of ovarian function in dogs and other carnivores. *Animal Reproduction* **6,** 172-193.

Concannon, P. W., Hansel, W. & McEntee, K. 1977. Changes in LH, progesterone and sexual behavior associated with preovulatory luteinization in the bitch. *Biology of Reproduction*, **17,** 604-613.

Concannon, P. W., Hansel, W. & Visek, W. J. 1975. The ovarian cycle of the bitch: plasma estrogen, LH, and progesterone. *Biology of Reproduction*, **13,** 112-121.

Coonan, T. J., Schwemm, C. A., Roemer, G. W., Garcelon, D. K. & Munson, L. 2005. Decline of an island fox subspecies to near extinction. *The Southwesteren Naturalist*, **50,** 32-41.

Creel, S. 2001. Social dominance and stress hormones. *Trends in Ecology and Evolution*, **16,** 493-497.

Creel, S. & Creel, N. M. 1995. Communal hunting and pack size in African wild dogs, *Lycaon pictus*. *Animal Behaviour*, **50,** 1325-1339.

Creel, S., Creel, N. M., Mills, M. G. L. & Montfort, S. L. 1997a. Rank and reproduction in cooperatively breeding African wild dogs: behavioral and endocrine correlates. *Behavioral Ecology* **8,** 298-306.

Creel, S., Creel, N. M. & Monfort, S. L. 1996. Social stress and dominance. *Nature*, **379,** 212.

Creel, S., Creel, N. M. & Monfort, S. L. 1997b. Radiocollaring and stress hormones in African wild dogs. *Conservation Biology*, **11,** 544-548.

Creel, S., Fox, J. E., Hardy, A., Sands, J., Garrot, B. & Peterson, R. O. 2002. Snowmobile activity and glucocorticoid stress responses in wolves and elk. *Conservation Biology*, **16,** 809-814.

Creel, S., Wildt, D. E. & Montfort, S. L. 1993. Aggression, reproduction and androgens in wild dwarf mongooses: a test of the challenge hypothesis. *The American Naturalist*, **141,** 816-825.

Creel, S. R. & Creel, N. M. 1991. Energetics, reproductive suppression and obligate communal breeding in carnivores. *Behavioral Ecology and Sociobiology*, **28,** 263-270.

Creel, S. R., Creel, N. M., Wildt, D. E. & Monfort, S. L. 1992. Behavioural and endocrine mechanisms of reproductive suppression in Serengeti dwarf mongooses. *Animal Behaviour*, **43,** 231-245.

Creel, S. R., Montfort, S. L., Wildt, D. E. & Waser, P. M. 1991. Spontaneous lactation is an adaptive result of pseudopregnancy. *Nature*, **351,** 660-662.

D'Agostino, J., Vaeth, G. F. & Henning, S. J. 1982. Diurnal rhythm of total and free concentrations of serum corticosterone in the rat. *Acta Endocrinologica*, **100,** 85-90.

De Ridder, E., Pinxten, R. & Eens, M. 2000. Experimental evidence of a testosterone-induced shift from paternal to mating behaviour in a facultatively polygynous songbird. *Behavioral Ecology and Sociobiology*, **49,** 24-30.

Delvonne, Y., Mansour, K. M. & C.F, F. 1996. Testosterone facilitates aggression by modulating vasopressin receptors in the hypothalamus. *Physiology and Behavior*, **60,** 25-29.

DeMatteo, K. E., Porton, I. J., Kleiman, D. G. & Asa, C. 2006. The effect of the male bush dog (*Speothos venaticus*) on the female reproductive cycle. *Journal of Mammalogy*, **87,** 723-732.

Derix, R., van Hooff, J., de Vries, H. & Wensing, J. 1993. Male and female mating competition in wolves: female suppression vs. male intervention. *Behaviour*, **127,** 141-174.

Dobson, A. & Lyles, A. 2000. Black-footed ferret recovery. *Science*, **288,** 985-988.

Doolan, S. P. & Macdonald, D. W. 1999. Co-operative rearing by slender-tailed meerkats (*Suricata suricatta*) in the Southern Kalahari. *Ethology*, **105,** 851-866.

Driscoll, C. A., Macdonald, D. W. & O'Brien, S. J. O. 2009. From wild animals to domestic pets, an evolutionary view of domestication. *Proceedings of the National Academy of Sciences*, **106,** 9971-9978.

Farstad, W. 1998. Reproduction in foxes: current research and future challenges. *Animal Reproduction Science*, **53,** 35-42.

Farstad, W. 2000a. Current state in biotechnology in canine and feline reproduction. *Animal Reproduction Science*, **60,** 375-387.

Farstad, W. 2000b. Assisted reproductive technology in canid species. *Theriogenology*, **53,** 175-186.

Farstad, W., Hyttel, P., Grøndahl, C., Mondain-Monval, M. & Smith, A. J. 1993. Fertilization and early embryonic development in the blue fox (*Alopex lagopus*). *Molecular Reproduction and Development*, **36,** 331-337.

Feldman, E. C. & Nelson, R. W. 1987. *Canine and feline endocrinology and reproduction*. Philadelphia: Saunders.

Finkler, H. & Terkel, J. 2010. Cortisol levels and aggression in neutered and intact free-roaming female cats living in urban social groups. *Physiology and Behavior*, **99,** 343-347.

Fitzgerald Industries International, I. 2010a. Polyclonal antibody data sheet rabbit anti estradiol 6. In: *Catalog number: 20R-ER005*. Acton, MA, USA.

Fitzgerald Industries International, I. 2010b. Polyclonal antibody data sheet, rabbit anti cortisol-3. In: *Catalog number: 20-CR50*. Concord, MA, USA.

Forsberg, M. & Madej, A. 1990. Effects of melatonin implants on plasma concentrations of testosterone, thyroxine and prolactin in the male silver fox (*Vulpes vulpes*). *Journal of Reproduction and Fertility*, **89,** 351-358.

Frantzen, M. A. J., Ferguson, J. W. H. & de Villiers, M. S. 2001. The conservation role of captive African wild dogs (*Lycaon pictus*). *Biological Conservation*, **100,** 253-260.

Fujita, S., Mitsunaga, F., Sugiura, H. & Shimizu, K. 2001. Measurement of urinary and fecal steroid metabolites during the ovarian cycle in captive and wild Japanese macaques, *Macaca fuscata*. *American Journal of Primatology*, **53,** 167-176.

Fusani, L. 2008. Testosterone control of male courtship in birds. *Hormones and Behavior*, **54,** 227-233.

FZS. 2010. Conservation of the Bale Mountains Ecosystem. Frankfurt Zoological Society, http://www.zgf.de/?projectId=5&id=65&language=en.

Garcia Pereira, R. J., Barbanti Duarte, J. M. & Negrão, J. A. 2005. Seasonal changes in fecal testosterone concentrations and their relationship to the reproductive behavior, antler cycle and grouping patterns in free-ranging male Pampas deer (*Ozotoceros bezoarticus bezoarticus*). *Theriogenology*, **63,** 2113-2125.

Garnier, J. N., Holt, W. V. & Watson, P. F. 2002. Non-invasive assessment of oestrus cycles and evaluation of reproductive seasonality in the female wild black rhinoceros (*Dicerons bicornis minor*). *Reproduction*, **123,** 877-889.

Geffen, E., Gompper, M. E., Gittleman, J. L., Luh, H., Macdonald, D. W. & Wayne, R. K. 1996. Size, life-history traits, and social organization in the Canidae: a reevaluation. *American Naturalist*, **147,** 140-160.

Girman, D. J., Mills, M. G. L., Geffen, E. & Wayne, R. K. 1997. A molecular genetic analysis of social structure, dispersal, and interpack relationships of the African wild dog (*Lycaon pictus*). *Behavioral Ecology and Sociobiology*, **40,** 187-198.

Gobello, C., De la Sota, R. L. & Goya, R. G. 2001. A review of canine pseudocyesis. *Reproduction of Domestic Animals*, **36,** 283-288.

Goodrowe, K. L., Hay, M. A., Platz, C. C., Behrns, S. K., Jones, M. H. & Waddell, W. T. 1998. Characteristics of fresh and frozen-thawed red wolf (*Canis rufus*) spermatozoa. *Animal Reproduction Science*, **53,** 299-308.

Gottelli, D., Marino, J., Sillero-Zubiri, C. & Funk, S. M. 2004. The effect of the last glacial age on speciation and population genetic structure of the endangered Ethiopian wolf (*Canis simensis*). *Molecular Ecology*, **13,** 2275-2286.

Gottelli, D., Sillero-Zubiri, C., Applebaum, G. D., Roy, M. S., Girman, D. J., Garcia-Moreno, J., Ostrander, E. A. & Wayne, R. K. 1994. Molecular genetics of the most

endangered canid: the Ethiopian wolf *Canis simensis*. *Molecular Ecology*, **3,** 301-312.

Goymann, W., East, M. L., Wachter, B., Höner, O. P., Möstl, E., Van't Hof, T. J. & Hofer, H. 2001. Social, state-dependent and environmental modulation of faecal corticosteroid levels in free-ranging female spotted hyenas. *Proceedings of the Royal Society, Series B,* **268,** 2453-2459.

Grafen, A. & Hails, R. 2002. *Modern statistics for the life sciences*. Oxford, United Kingdom: Oxford University Press.

Graham, L. H. 2004. Non-invasive monitoring of reproduction in zoo and wildlife species. *Annual Review of Biomedical Sciences*, **6,** 91-98.

Gubernick, D. J. & Teferi, T. 2000. Adaptive significance of male paternal care in a monogamous mammal. *Proceedings of the Royal Society Series B: Biological Sciences*, **267,** 147-150.

Gudermuth, D. F., Concannon, P. W., Daels, P. F. & Lasley, B. L. 1998. Pregnancy-specific elevations in fecal concentrations of estradiol, testosterone, and progesterone in the domestic dog (*Canis familiaris*). *Theriogenology*, **50,** 237-248.

Gusset, M., Ryan, S. J., Hofmeyr, M., van Dyk, G., Davies-Mostert, H., T, Graf, J. A., Owen, C., Szykman, M., Macdonald, D. W., Montfort, S. L., Wildt, D. E., Maddock, A. H., Mills, M. G. L., Slotow, R. & Somers, M. J. 2008. Efforts going to the dogs? Evaluating attempts to re-introduce endangered wild dogs in South Africa. *Journal of Applied Ecology*, **45,** 100-108.

Hadley, M. E. 2000. *Endocrinology*. Upper Saddle River, NJ: Prentice-Hall Inc.

Harding, C. F. 1981. Social modulations of circulating hormone levels in the male. *American Zoologist*, **21,** 223-231.

Hart, B. E. 1983. Role of testosterone secretion and penile reflexes in sexual behavior and sperm competition in male rats: a theoretical contribution. *Physiology and Behavior*, **31,** 823-827.

Haydon, D. T., Randall, D. A., Matthews, L., Knobel, D. L., Tallents, L. A., Gravenor, M. B., Williams, S. D., Pollinger, J. P., Cleaveland, S., Woolhouse, M. E. J., Sillero-Zubiri, C., Marino, J., MacDonald, D. W. & Laurenson, M. K. 2006. Low-coverage vaccination strategies for the conservation of endangered species. *Nature*, **443,** 692-695.

Heape, W. 1897. The artificial insemination of mammals and subsequent possible fertilisation or impregnation of their ova. *Proceedings of the Royal Society of London*, **61,** 52-63.

Hedrick, P. W. & Fredickson, R. J. 2008. Captive breeding and the reintroduction of Mexican and red wolves. *Molecular Ecology*, **17,** 344-350.

Helle, E. & Kauhala, K. 1995. Reproduction in the raccoon dog in Finland. *Journal of Mammalogy*, **76,** 1036-1046.

Hendrickson, J. r. 1958. The green sea turtle *Chelonia mydas* (Linn.) in Malaya and Sarawak. *Journal of Zoology*, **130,** 455-535.

Hillman, J. C. 1988. The Bale Mountains National Park area, Southeast Ethiopia, and its management. *Mountain Research and Development*, **8,** 253-258.

Hoffmann, B., Büsges, F., Engel, E., Kowalewski, M. P. & Papa, P. 2004. Regulation of corpus luteum function in the bitch. *Reproduction of Domestic Animals*, **39,** 232-240.

Hoffmann, M., Riesenbeck, A, Klein, R. 1996. Reproductive endocrinology of bitches. *Animal Reproduction Science*, **42,** 275-288.

Hradecky, P. 1985. Possible pheromonal regulation of reproduction in wild carnivores. *Journal of Chemical Ecology*, **11,** 241-250.

IUCN. 2010. Red list of threatened species. In: *www.iucn.org*.

Jennions, M. D. & Macdonald, D. W. 1994. Cooperative breeding in mammals. *Trends in Ecology and Evolution*, **9,** 89-93.

Jöchle, W. & Andersen, A. C. 1976. The estrous cycle in the dog: a review. *Theriogenology*, **7,** 113-140.

Joffre, M. 1977. Relationship between testicular blood flow, testosterone secretion and spermatogenic activity in young and adult wild red foxes (*Vulpes vulpes*). *Journal of Reproduction and Fertility*, **51,** 35-40.

Johnson, N., Mansfield, K. L., Marston, D. A., Wilson, C., Goddard, T., Selden, D., Hemson, G., Edea, L., Van Kesteren, F., Shiferaw, F., Stewart, A. E., Sillero-Zubiri, C. & Fooks, A. R. 2010. A new outbreak of rabies in rare Ethiopian wolves (*Canis simensis*). *Archives of Virology*, **155,** 1175-1177.

Johnston, S. D., Ward, D., Lemon, J., Gunn, I., MacCallum, C. A., Keeley, T. & Blyde, D. 2007. Studies of male reproduction in captive African wild dogs (*Lycaon pictus*). *Animal Reproduction Science*, **100,** 338-355.

Kauhala, K. & Saeki, M. 2004a. Finnish and Japanese raccoon dogs - on the road to speciation? In: *Biology and Conservation of Wild Canids* (Ed. by Macdonald, D. W. & Sillero-Zubiri, C.). Oxford, UK: Oxford University Press.

Kauhala, K. & Saeki, N. 2004b. Raccoon dog, *Nyctereutes procynoides*. In: *Canids: foxes, wolves, jackals and dogs. Status Survey and Conservation Action Plan* (Ed. by Sillero-Zubiri, C., Hoffmann, M. & Macdonald, D. W.). Gland, Swizerland ans Cambridge, UK: IUCN/SSC Canid Specialist Group.

Kemppainen, R. J. & Sartin, J. L. 1984. Evidence for episodic but not circadian activity in plasma concentrations of adrenocorticotrophin, cortisol and thyroxine in dogs. *Journal of Endocrinology*, **103,** 219-226.

Kennelly, J. J. & Johns, B. E. 1976. The estrous cycle of coyotes. *Journal of Wildlife Management*, **40,** 272-277.

Khan, M. Z., Altman, J., Isani, S. S. & Yu, J. 2002. A matter of time: evaluating the storage of fecal samples for steroid analysis. *General and Comparative Endocrinology*, **128,** 57-64.

Khan, M. Z., McNabb, F. M. A., Walters, J. R. & Sharp, P. J. 2001. Patterns of testosterone and prolactin concentrations and reproductive behaviour of helpers and breeders in the cooperatively breeding red-cockaded woodpecker (*Picoides borealis*). *Hormones and Behavior*, **40,** 1-13.

Kleiman, D. G. 1989. Reintroduction of captive mammals for conservation. *BioScience*, **39,** 152-161.

Kolevska, J., Brunclik, V. & Svoboda, M. 2003. Circadian rhythm of cortisol secretion in dogs of different daily activities. *Acta Veterianaria Brunenses*, **72,** 599-605.

Komers, P. E. 1996. Obligate monogamy without paternal care in Kirk's dikdik. *Animal Behaviour*, **51,** 131-140.

Kooistra, H. S. & Okkens, A. C. 2001. Role of changes in the pulsatile secretion pattern of FSH in initiation of ovarian folliculogenesis in bitches. In: *Advances in reproduction in dogs, cats and exotic carnivores : proceedings of the Fourth International Symposium on Canine and Feline Reproduction* (Ed. by Concannon, P. W.). Oslo, Norway: Journal of Reproduction and Fertility.

Kooistra, H. S., Okkens, A. C., M.M, B., Popp-Snijders, C., van Haaften, B., Dieleman, S. J. & J, S. 1999. Concurrent pulsatile secretion of luteinizing hormone and follicle-

stimulating hormone during different phases of the estrous cycle and anestrus in bitches. *Biology of Reproduction*, **60,** 65-71.

Korhonen, H. & Alasuutari, S. 1992. Sleeping distance in relation to sexual state in the Arctic blue fox. *Reproduction Nutrition Development*, **32,** 123-127.

Kreeger, T. J. 2003. The internal wolf: physiology, pathology and pharmacology. In: *Wolves: behavior, ecology and conservation* (Ed. by Mech, L. D. & Boitani, L.), pp. 192-217. Chicago: University of Chicago Press.

Laurenson, K., Sillero-Zubiri, C., Thompson, H., Shiferaw, F., Thirgood, S. & Malcolm, J. 1998. Disease as a threat to endangered species: Ethiopian wolves, domestic dogs and canine pathogens. *Animal Conservation*, **1,** 273-280.

Louch, C. D. & Higginbotham, M. 1967. The relation between social rank and plasma corticosterone in mice. *General and Comparative Endocrinology*, **8,** 441-444.

Lynch, J. W., Ziegler, T. E. & Strier, K. B. 2002. Individual and seasonal variation in fecal testosterone and cortisol levels of wild male tufted capuchin monkeys, *Cebus apella nigritus*. *Hormones and Behavior*, **41,** 275-287.

Macdonald, D. W. 1980. Social factors affecting reproduction amongst red foxes (*Vulpes vulpes* L., 1758). In: *The red fox: symposium on behaviour and ecology* (Ed. by Zimen, E.). The Hague: Dr. W. Junk bv publishers.

Macdonald, D. W., Creel, S. & Mills, M. G. L. 2004. Canid society. In: *The biology and conservation of wild canids* (Ed. by Macdonald, D. W. & Sillero-Zubiri, C.). Oxford, UK: Oxford University Press.

Macdonald, D. W. & Sillero-Zubiri, C. 2004. Biology and conservation of wild canids. (Ed. by MacDonald, D. W. & Sillero-Zubiri, C.). Oxford: Oxford University Press.

Malcolm, J. 1997. The diet of Ethiopian wolf (*Canis simensis* Ruppell) from a grassland area of the Bale Mountains, Ethiopia. *African Journal of Ecology*, **35,** 162-164.

Marino, J. 2003a. Spatial ecology of the Ethiopian wolf, *Canis simensis*. PhD Thesis, *Department of Zoology*. Oxford: University of Oxford.

Marino, J. 2003b. Threatened Ethiopian wolves persist in small isolated Afroalpine enclaves. *Oryx*, **37,** 62-71.

Marino, J., Sillero-Zubiri, C., Johnson, P. J. & Macdonald, D. W. submitted. Group territoriality brings ecological gains to expansionist Ethiopian wolves *Behavioral Ecology and Sociobiology*.

Marino, J., Sillero-Zubiri, C. & MacDonald, D. W. 2006. Trends, dynamics and resilience of an Ethiopian wolf population. *Animal Conservation*, **9,** 49-58.

Maurel, D. & Boissin, J. 1981. Plasma thyroxine and testosterone levels in the red fox (*Vulpes vulpes* L.) during the annual cycle. *General and Comparative Endocrinology*, **43,** 402-404.

May, R. M. 1986. The cautionary tale of the black-footed ferret. *Nature*, **320,** 13-14.

McNatty, K. P., Cashmore, M. & Young, A. 1972. Diurnal variation in plasma cortisol levels in sheep. *Journal of Endocrinology*, **54,** 361-362.

McNutt, J. W. 1996. Sex-biased dispersal in African wild ogs, *Lycaon pictus*. *Animal Behaviour*, **52,** 1067-1077.

Meyers-Wallen, V. N. 2007. Unusual and abnormal canine estrous cycles. *Theriogenology*, **68,** 1205-1210.

Millspaugh, J. J. & Washburn, B. E. 2004. Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. *General and Comparative Endocrinology*, **138,** 189-199.

Millspaugh, J. J., Washburn, B. E., Milanick, M. A., Slotow, R. & van Dyk, G. 2003. Effects of heat and chemical treatments on fecal glucocorticoid measurements: implications for sample transport. *Wildlife Society Bulletin*, **31,** 399-406.

Minter, L. J. & DeLiberti, T. J. 2008. Seasonal variation in serum testosterone, testicular volume, and semen characteristics in the coyote (*Canis latrans*). *Theriogenology*, **69,** 946-952.

Møller, A. P. 1973. Progesterone concentrations in the peripheral plasma of the blue fox (*Alopex lagopus*) during pregnancy and the oestrous cycle. *Journal of Endocrinology*, **59,** 429-438.

Møller, A. P. 1988. Paternity and paternal care in the swallow, *Hirundo rustica*. *Animal Behaviour*, **36,** 996-1005.

Møller, O. M., Mondain-Monval, M., Smith, A., Metzget, E. & Scholler, R. 1984. Temporal relationships between hormonal concentrations and the electrical resistance of the vaginal tract of blue foxes (*Alopex lagopus*) at pro-oestrus and oestrus. *Journal of Reproduction and Fertility*, **70,** 15-24.

Mondain-Monval, M., Bonnin, M., Canivenc, R. & Scholler, R. 1984. Heterologous radioimmunoassay of fox LH: levels during the reproductive season and the anoestrus of the red fox (*Vulpes vulpes* L.). *General and Comparative Endocrinology*, **55,** 125-132

Mondain-Monval, M., Dutourner, B., Bonnin-Laffargue, M., Canivenc, R. & Scholler, R. 1977. Ovarian activity during the anoestrus and the reproductive season of the red fox (*Vulpes vulpes* L.). *Journal of Steroid Biochemistry*, **8,** 761-769.

Montfort, S. L., Mashburn, K. L., Brewer, B. A. & Creel, S. 1998. Evaluating adrenal activity in African wild dogs (*Lycaon pictus*) by fecal corticosteroid analysis. *Journal of Zoo and Wildlife Medicine*, **29,** 129-133.

Montfort, S. L., Wasser, S. K., Mashburn, K. L., Burke, M., Brewer, B. A. & Creel, S. R. 1997. Steroid Metabolism and Validation of Noninvasive Endocrine Monitoring in the African Wild Dog (*Lycaon pictus*). *Zoo Biology*, **16,** 533-548.

Moss, A. M., Clutton-Brock, T. & Monfort, S. L. 2001. Longitudonal gonadal steroid excretion in free-living male and female meerkats (*Suricata suricatta*). *General and Comparative Endocrinology,* **122,** 158-171.

Möstl, E. & Palme, R. 2002. Hormones as indicators of stress. *Domestic Animal Endocrinology*, **23,** 64-74.

Muller, M. N. & Wrangham, R. W. 2004. Dominance, aggression and testosterone in wild chimpanzees: a test of the 'challenge hypothesis'. *Animal Behaviour*, **67,** 113-123.

Nichols, K. 2009. A non-invasive assessment of the oestrus cycle of the Visayan warty pig (*Sus cebifrons negrinus*) held in captivity in Europe. MSc thesis, *Centre for Reproductive Biology*. Edinburgh: University of Edinburgh.

OEM-Concepts. 2010. Antibody specification sheet rabbit anti testosterone-19. In: *Catalog number: RS-S07*. Saco, ME, USA.

Okkens, A. C., Bevers, M. M., Dieleman, S. J., van Haaften, B. & van Sluijs, F. J. 1992. Fertility problems in the bitch. *Animal Reproduction Science*, **28,** 379-387.

Olson, P. N., Bowen, R. A., Behrendt, M. D., Olson, J. D. & Nett, T. M. 1984. Concentrations of progesterone and luteinizing hormone in the serum of diestrous bitches before and after hysterectomy. *American Journal of Veterinary Research*, **45,** 149-153.

Ortega-Pacheco, A., Segura-Correa, J. C., Bolio-Gonzaes, M. E., Jiménez-Coello, J. & Linde Forsberg, C. 2006. Reproductive patterns of stray male dogs in the tropics. *Theriogenology*, **66,** 2084-2090.

Ostrowski, S., Blanvillain, C., Mesochina, P., Ismail, K. & Schwarzenberger, F. 2005. Monitoring reproductive steroids in feces of Arabian oryx: toward a non-invasive method to predict reproductive status in the wild. *Wildlife Society Bulletin*, **33,** 967-973.

Palme, R., Fischer, P., Schildorfer, H. & Ismail, M. N. 1996. Excretion of 14C-steroid hormones via feaces and urine in domestic livestock. *Animal Reproduction Science*, **43,** 43-63.

Palme, R. & Möstl, E. 1993. Biotin-streptavidin enzyme immunoassay for the determination of oestrogens and androgens in boar faeces. In: *Proceedings of the 5th Symposium on the Analysis of Steroids* (Ed. by S, G.). Szombathely, Hungary.

Palme, R. & Möstl, E. 1996. Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in bloodq. In: *Proceesings of the conference on physiology and ethology of wild and zoo animals* (Ed. by Fischer, G.). Berlin, Germany.

Pantex. 2010. Polyclonal antibody datasheet rabbit anti progesterone. In: *Catalog number: PROG-#51C*. Santa Monica, CA, USA.

Paris, M. C. J., White, A., Reiss, A., West, M. & Schwarzenberger, F. 2002. Faecal progesterone metabolites and behavioural observations for the non-invasive assessment of oestrous cycles in the common wombat (*Vombatus ursinus*) and the southern hairy-nosed wombat (*Lasiorhinus latifrons*). *Animal Reproduction Science*, **72,** 245-257.

Pettitt, B. A., Wheaton, C. J. & Waterman, J. M. 2007. Effects of storage treatment on fecal steroid hormone concentrations of a rodent, the Cape ground squirrel (*Xerus inauris*). *General and Comparative Endocrinology*, **150,** 1-11.

Phemister, R. D., Holst, P. A., Spano, J. S. & Hopwood, M. L. 1973. Time of ovulation in the Beagle bitch. *Biology of Reproduction*, **8,** 74-82.

Pierce, B. N., Clarke, I. J., Turner, A. I., Rivalland, E. T. A. & Tilbrook, A. J. 2009. Cortisol disrupts the ability of estradiol-17β to induce the LH surge in ovariectomized ewes. *Domestic Animal Endocrinology*, **36,** 202-208.

Plant, T. M. 1981. Time courses of concentrations of circulating gonadotropin, prolactin, testosterone, and cortisol in adult male rhesus monkeys (*Macaca mulatta*) throughout the 24 h light-dark cycle. *Biology of Reproduction*, **25,** 244-252.

Plotka, E. D., Seal, U. S., Verme, L. J. & Ozoga, J. J. 1983. The adrenal gland in white-tailed deer: a significant source of progesterone. *Journal of Wildlife Management*, **47,** 38-44.

Porton, I. J. 1983. Bush dog urine-marking: its role in pair formation and maintenance. *Animal Behaviour*, **31,** 1061-1069.

Porton, I. J., Kleiman, D. G. & Rodden, M. D. 1987. Aseasonality of bush dog reproduction and the influence of social factors on the estrous cycle. *Journal of Mammalogy*, **64,** 867-871.

Qiang, W., Murase, T. & Subota, T. 2003. Seasonal changes in spermatogenesis and testicular steroidogenesis in wild male raccoon dogs (*Nyctereutes procynoides*). *Journal of Veterinary Medicinal Science*, **65,** 1087-1092.

Randall, D. 2006. Determinants of genetic variation in the Ethiopian wolf, *Canis simensis*. In: *Wildlife Conservation Research Unit, Department of Zoology*. Oxford: University of Oxford.

Randall, D., Pollinger, J. P., Wayne, R. K., Tallents, L. A., Johnson, P. J. & MacDonald, D. W. 2007. Inbreeding is reduced by female-biased dispersal and mating behavior in Ethiopian wolves. *Behavioral Ecology*, **18,** 579-589.

Randall, D., Williams, S. D., Kuzmin, I. V., Rupprecht, E., Tallents, L. A., Tefera, Z., Argaw, K., Shiferaw, F., Knobel, D. L., Sillero-Zubiri, C. & Laurenson, M. K. 2004. Rabies in endangered Ethiopian wolves. *Emerging Infectious Diseases*, **10,** 2214-2217.

Roemer, G. W., Coonan, T. J., Munson, L. & Wayne, R. K. 2004. Island fox, *Urocyon littoralis*. In: *Canids: foxes, wolves, jackals and dogs. Status Survey and Conservation Action Plan* (Ed. by Sillero-Zubiri, C., Hoffmann, M. & Macdonald, D. W.). Gland, Swtitzerland and Cambridge, UK: IUCN/SSC Canid Specialist Group.

Romero, L. M. 2002. Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. *General and Comparative Endocrinology*, **128,** 1-24.

Rosado, B., Garcia-Belenguer, S., Leon, M., Chacon, G., Villegas, A. & Palacio, J. 2010. Blood concentrations of serotonin, cortisol and dehydroepiandrosterone in aggressive dogs. *Applied Animal Behaviour Science*, **123,** 124-130.

Rudert, S., Brown, J. L., Gansloβer, U., Möbius, G. & Songsasen, N. 2010. Activity pattern, reproductive behaviors and gonadal hormones in the raccoon dog (*Nyctereures procyonoides*). *Zoo Biology*, **29,** 1-15.

Safar-Hermann, N., Ismail, M. N., Choi, H. S., Möstl, E. & Bamberg, E. 1987. Pregnancy diagnosis in zoo animals by estrogen determination in feces. *Zoo Biology*, **6,** 189-193.

Saltzman, W., Schutz-Darken, N. J., Wegner, F. H., Wittwer, D. J. & Abbott, D. H. 1998. Suppression of cortisol levels in subordinate female marmosets: reproductive and social contributions. *Hormones and Behavior*, **33,** 58-74.

Sands, J. & Creel, S. 2004. Social dominance, aggression and faecal glucocorticoid levels in a wild population of wolves, *Canis lupus*. *Animal Behaviour*, **67,** 387-396.

Sanson, G., Brown, J. L. & Farstad, W. 2005. Non-invasive faecal steroid monitoring of ovarian and adrenal activity in farmed blue fox (*Alopex lagopus*) females during late pregnancy, parturition and lactation onset. *Animal Reproduction Science*, **87,** 309-319.

Sapolsky, R. M., Alberts, S. C. & Altmann, J. 1997. Hypercortisolism associated with social subordinance or social isolation among wild baboons. *Archives of General Psychiatry*, **54,** 1137-1143.

Schatz, S. & Palme, R. 2001. Measurement of faecal cortisol metabolites in cats and dogs: a non-invasive method for evaluating adrenocortical function. *Veterinary Research Communications*, **25,** 271-287.

Schenkel, R. 1967. Submission: its features and function in the wolf and dog. *American Zoologist*, **7,** 319.

Schuurs, A. H. W. M. & van Weemen, B. K. 1980. Enzyme-Immunoassay: a powerful analytical tool. *Journal of Immunoassay and immunochemistry*, **1,** 229-249.

Schwarzenberger, F. 2007. The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species. *International Zoo Yearbook*, **41,** 1-23.

Schwarzenberger, F., Rietschel, W., Vahala, J., Holeckova, D., Thomas, P., Maltzan, J., Baumgartner, K. & Schaftenaar, W. 2000. Fecal progesterone, estrogen, and androgen metabolites for noninvasive monitoring of reproductive function in the female Indian rhinoceros, *Rhinoceros unicornis*. *General and Comparative Endocrinology*, **119,** 300-307.

Schwarzenberger, F., Tomášová, K., Holeckova, D., Matern, B. & Möstl, E. 1996. Measurement of fecal steroids in the black rhinocerus (*Diceros bicornis*) using group-specific enzyme immunoassays for 20-oxo-pregnanes. *Zoo Biology*, **15,** 159-171.

Seal, U. S., Plotka, E. D., Packard, J. M. & Mech, L. D. 1979. Endocrine correlates of reproduction in the wolf. 1. Serum progesterone, estradiol and LH during the estrous cycle. *Biology of Reproduction*, **21,** 1057-1066.

Shideler, S. E., Munro, C. J., Johl, H. K., Taylor, H. W. & Lasley, B. L. 1995. Urine and fecal sample collection on filter paper for ovarian hormone evaluations. *American Journal of Primatology*, **37,** 305-315.

Sillero-Zubiri, C. 1994. Behavioural ecology of the Ethiopian wolf, *Canis simensis*. PhD thesis, *Wildlife Conservation Research Unit, Department of Zoology*. Oxford: University of Oxford.

Sillero-Zubiri, C. & Gottelli, D. 1994. *Canis simensis*. *Mammalian Species*, **485,** 1-6.

Sillero-Zubiri, C. & Gottelli, D. 1995a. Diet and feeding behavior of Ethiopian wolves (*Canis simensis*). *Journal of Mammalogy*, **76,** 531-541.

Sillero-Zubiri, C. & Gottelli, D. 1995b. Spatial organization in the Ethiopian wolf *Canis simensis*: large packs and stable home ranges. *Journal of Zoology*, **237,** 65-81.

Sillero-Zubiri, C., Gottelli, D. & Macdonald, D. W. 1996a. Male philopatry, extra-pack copulations and inbreeding avoidance in Ethiopian wolves (*Canis simensis*). *Behavioral Ecology and Sociobiology*, **38,** 331-340.

Sillero-Zubiri, C., Hoffmann, M. & Macdonald, D. W. 2004a. Canids: Foxes, wolves, jackals and dogs. In: *IUCN Status Survey and Conservation Action Plan* (Ed. by Sillero-Zubiri, C., Hoffman, M. & MacDonald, D. W.). Gland, Switzerland and Cambridge: IUCN/SSc Canid Specialist Group.

Sillero-Zubiri, C., Johnson, P. J. & Macdonald, D. W. 1998. A hypothesis for breeding synchrony in Ethiopian wolves (*Canis simensis*). *Journal of Mammalogy*, **79,** 853-858.

Sillero-Zubiri, C., King, A. A. & Macdonald, D. W. 1996b. Rabies and mortality in Ethiopian wolves (*Canis simensis*). *Journal of Wildlife Diseases*, **32,** 80-86.

Sillero-Zubiri, C. & Macdonald, D. W. 1997. The Ethiopian wolf - Status Survey and Conservation Action Plan. In: *IUCN/SSC Canid Specialist Group* (Ed. by Sillero-Zubiri, C. & MacDonald, D. W.). Gland,Switzerland and Cambridge, UK: IUCN.

Sillero-Zubiri, C. & Macdonald, D. W. 1998. Scent-marking and territorial behaviour of Ethiopian wolves *Canis simensis*. *Journal of Zoology*, **245,** 351-361.

Sillero-Zubiri, C., Marino, J., Gottelli, D. & Macdonald, D. W. 2004b. Ethiopian wolves: Afroalpine ecology, solitary foraging, and intense sociality amongst Ethiopian wolves. In: *Biology and conservation of wild canids* (Ed. by Macdonald, D. W. & Sillero-Zubiri, C.). Oxford, UK: Oxford University Press.

Smith, A. J., Clausen, O. P. F., Kirkhus, B., Jahnsent, T., Møller, O. M. & Hansson, V. 1984. Seasonal changes in spermatogenesis in the blue fox (*Alopex lagopus*), quantified by DNA flow cytometry and measurement of soluble Mn^{2+}-dependent adenylate cyclase activity. *Journal of Reproduction and Fertility*, **72,** 453-461.

Smith, A. J., Mondain-Monval, M., Møller, O. M., Scholler, R. & Hansson, V. 1985. Seasonal variations of LH, prolactin, androstenedione, testosterone and testicular FSH binding in the male blue fox (*Alopex lagopus*). *Journal of Reproduction and Fertility*, **74,** 449-458.

Smith, M. S. & McDonald, L. E. 1974. Serum levels of luteinizing hormone and progesterone during the estrous cycle, pseudopregancy, and pregnancy in the dog. *Endocrinology*, **94**.

Snyder, N. F. R., Derrickson, S. R., Beissinger, S. R., Wiley, J. W., Smith, T. S., Toone, W. D. & Miller, B. 1996. Limitations of captive breeding in endangered species recovery. *Conservation Biology*, **10,** 338-348.

Sokal, R.P. & Rohlf, F.J. 1981. *Biometry*, New York, U.S.A, W.H. Freeman and Company.

Solomon, N. G. & French, J. A. 1997. *Cooperative breeding in mammals*. Cambridge: Cambridge University Press.

Songsasen, N., Rodden, M. D., Brown, J. L. & Wildt, D. E. 2006. Patterns of fecal gonadal hormone metabolites in the maned wolf (*Chrysocyon brachyurus*). *Theriogenology*, **66,** 1743-1750.

Soto-Gamboa, M., Villalón, M. & Bozinovic, F. 2005. Social cues and hormone levels in male *Octoden degus* (Rodentia): a field test of the Challenge Hypothesis. *Hormones and Behavior*, **47,** 311-318.

Stephen, A. M. & Cummings, J. H. 1980. The microbial contribution to human faecal mass. *Journal of Medical Microbiology*, **13,** 45-56.

Stephens, P. A., d'Sa, C. A., Sillero-Zubiri, C. & Leader-Williams, N. 2001. Impact of livestock and settlement on the large mammalian wildlife of Bale Mountains National Park, southern Ethiopia *Biological Conservation*, **100,** 307-322.

Stewart, A., Gordon, C. & Marino, J. 2010. Ethiopian Wolf Conservation Programme Annual Report. www.ethiopianwolf.org.

Strier, K. B., Ziegler, T. E. & Wittwer, D. J. 1999. Seasonal and social correlates of fecal testosterone and cortisol levels in wild male muriquis (*Brachyteles arachnoides*). *Hormones and Behavior*, **35,** 125-134.

Tallents, L. A. 2007. Determinants of reproductive success in Ethiopian wolves. PhD thesis, *Wildlife Conservation Research Unit, Department of Zoology*. Oxford: University of Oxford.

Terio, K. A., Brown, J. L., Moreland, R. & Munson, L. 2002. Comparison of different drying and storage methods on quantifiable concentrations of fecal steroids in the cheetah. *Zoo Biology*, **21,** 215-222.

Thomassen, R. & Farstad, W. 2009. Artificial insemination in canids: a useful tool in breeding and conservation. *Theriogenology*, **71,** 190-199.

Thun, R., Eggenberger, E. & Zerobin, K. 1990. 24-Hour profiles of plasma cortisol and testosterone in the male dog: absence of circadian rythmicity, seasonal influence and hormonal interrelationships. *Reproduction of Domestic Animals*, **25,** 68-77.

Thurber, J. M. & Peterson, R. O. 1993. Effects of population density and pack size on the foraging ecology of gray wolves. *Journal of Mammalogy*, **74,** 879-889.

Tsutsui, T. 1983. Effects of ovariectomy and progesterone treatment on the maintenance of pregnancy in bitches. *Japanese Journal of Veterinary Science*, **45,** 47-51.

Valdespino, C., Asa, C. & Bauman, J. E. 2002. Estrous cycles, copulation, and pregnancy in the fennec fox (*Vulpes zerda*). *Journal of Mammalogy*, **83,** 99-109.

Valtonen, M. H., Rajakoski, E. J. & Lähteenmäki, P. 1978. Levels of oestrogen and progesterone in the plasma of the raccoon dog (*Nyctereutes procynoides*) during oestrus and pregnancy. *Journal of Endocrinology*, **76,** 549-550.

Valtonen, M. H., Rajakoski, E. J. & Mäkelä, J. I. 1977. Reproductive features in the female raccoon dog (*Nyctereutes procyonoides*). *Journal of Reproduction and Fertility*, **51,** 517-518.

van der Molen, H. J., van Beurden, W. M. O., Blankenstein, M. A., de Boer, W., Cooke, B. A., Grootegoed, J. A., Janszen, F. H. A., de Jong, F. H., Mulder, E. & Rommerts, F. F. G. 1979. The testis: biochemical actions of trophic hormones and steroids on steroid on steroid production and spermatogenesis. *Journal of Steroid Biochemistry*, **11,** 13-18.

Velloso, A. L., Wasser, S. K., Montfort, S. L. & Dietz, J. M. 1998. Longitudinal fecal steroid secretion in maned wolves (*Chrysocyon brachyrus*). *General and Comparative Endocrinology*, **112,** 96-107.

Verhage, H. G., Beamer, N. B. & Brenner, R. M. 1976. Plasma levels of estradiol and progesterone in the cat during polyestrus, pregnancy and pseudopregnancy. *Biology of Reproduction*, **14,** 579-585.

Walker, S. L., Waddell, W. T. & Goodrowe, K. L. 2002. Reproductive endocrine patterns in captive female and male red wolves (*Canis rufus*) assessed by fecal and serum hormone analysis. *Zoo Biology*, **21,** 321-335.

Wasser, S. K. 1996. Reproductive control in wild baboons measured by fecal steroids. *Biology of Reproduction*, **55,** 393-399.

Wasser, S. K., De Lemos Velloso, A. & Rodden, M. D. 1995. Using fecal steroids to evaluate reproductive function in female maned wolves. *The Journal of Wildlife Management*, **59,** 889-894.

Wasser, S. K., Montfort, S. L. & Wildt, D. E. 1991. Rapid extraction of faecal steroids for measuring reproductive cyclicity and early pregnancy in free-ranging yellow baboons (*Papio cynocephalus cynocephalus*). *Journal of Reproduction and Fertility*, **92,** 415-423.

Wasser, S. K., Papageorge, S., Foley, C. & Brown, J. L. 1996. Excretory fate of estradiol and progesterone in the African elephant (*Loxodonta africana*) and patterns of fecal steroid concentrations throughout the estrous cycle. *General and Comparative Endocrinology*, **102,** 255-262.

Wasser, S. K., Risler, L. & Steiner, R. A. 1988. Excreted steroids in primate feces over the menstrual cycle and pregnancy. *Biology of Reproduction*, **39,** 862-872.

Weng, Q., Medan, M. S., M, X., Tsubota, T., Watanabe, G. & Taya, K. 2006. Seasonal changes in immunolocalization of inhibin/activin subunits and testicular activity in wild male raccoon dogs (*Nyctereutes procyonoides*). *Journal of Reproduction and Development*, **52,** 503-510.

Whitten, P. L., Brockman, D. K. & Stavisky, R. C. 1998. Recent advances in noninvasive techniques to monitor hormone-behavior interactions. *Yearbook of Physical Anthropology*, **41,** 1-23.

Wildt, D. E., Ellis, S. & Howard, J. G. 2001. Linkage of reproduction sciences: from 'quick fix' to 'integrated' conservation. In: *Advances in reproduction in dogs, cats and exotic carnivores : proceedings of the Fourth International Symposium on Canine and Feline Reproduction* (Ed. by Concannon, P. W.). Oslo, Norway: Journal of Reproduction and Fertility.

Wildt, D. E., Panko, W. B., Chakraborty, P. K. & Seager, S. W. J. 1979. Relationship of serum estrone, estradiol-17β and progesterone to LH, sexual behavior and time of ovulation in the bitch. *Biology of Reproduction*, **20,** 648-658.

Wildt, D. E. & Wemmer, C. 1999. Sex and wildlife: the role of reproductive science in conservation. *Biodiversity and Conservation*, **8,** 965-976.

Williams, E. S., Thorne, E. T., Appel, M. J. G. & Belitsky, D. W. 1988. Canine distemper in black-footed ferrets (Mustela nigripes) from Wyoming. *Journal of Wildlife Diseases*, **24,** 385-398.

Wilson, J. D., George, F. W. & Griffin, J. E. 1981. The hormonal control of sexual development. *Science*, **211,** 1278-1284.

Wingfield, J. C. 1984. Androgens and mating systems: testosterone-induced polygyny in normally monogamous birds. *The Auk*, **101,** 665-671.

Wingfield, J. C., Hegner, R. E., Dufty Jr, A. F. & Ball, G. F. 1990. The "Challenge Hypothesis": theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. *American Naturalist*, **136,** 829-846.

Wisdom, B. G. 1976. Enzyme-Immunoassay. *Clinical chemistry*, **22,** 1243-1255.

Woodroffe, R., McNutt, J. W. & Mills, M. G. L. 2004. African wild dog, *Lycaon pictus*. In: *Canids: foxes, wolves, jackals and dogs. Status Survey and Conservation Action Plan* (Ed. by Sillero-Zubiri, C., Hoffmann, M. & Macdonald, D. W.). Gland, Swizerland ans Cambridge, UK: IUCN/SSC Canid Specialist Group.

Xiao, Y., Forsberg, M., Laitinen, J. T. & Valtonen, M. 1995. Effects of melatonin implants in spring on testicular regression and moulting in adult male raccoon dogs (*Nyctereutes procynoides*). *Journal of Reproduction and Fertility*, **105,** 9-15.

Yahnke, C. J., Johnson, W. E., Geffen, E., Smith, D., Hertel, F., Roy, M. S., Bonacic, C. F., Fuller, T. K., van Valkenburgh, B. & Wayne, R. K. 1996. Darwin's fox: a distinct endangered species in a vanishing habitat. *Conservation Biology*, **10,** 366-375.

Yalden, D. W. & Largen, M. J. 1992. The endemic mammals of Ethiopia. *Mammal Review*, **22,** 115-150.

Young, K. M., Walker, S. L., Lanthier, C., Waddell, W. T., Monfort, S. L. & Brown, J. L. 2004. Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analyses. *General and Comparative Endocrinology*, **137,** 148-165.

Ziegler, T. E., Scheffler, G. & Snowdon, C. T. 1995. The relationship of cortisol levels to social environment and reproductive functioning in female cotton-top tamarins, *Saguinus oedipus*. *Hormones and Behavior*, **29,** 407-424.

Zirkin, B. R. 1998. Spermatogenesis: its regulation by testosterone and FSH. *Cell and Developmental Biology*, **9,** 417-421.

Zirkin, B. R., Santullu, R., Awoniyi, C. A. & Ewing, L. L. 1989. Maintenance of advanced spermatogenic cells in the adult rat testis: quantitative relationship to testosterone concentration within the testis. *Endocrinology*, **124,** 3043-3049.

Appendix I: A new outbreak of rabies in rare Ethiopian wolves, *Canis simensis*

Appendix II: Mating observations recorded as part of this study

Field season	Pack	Date	Estrous day	Female	Female status	Male	Male status	Behaviour	Notes
1	Sodota	04/09/2007	-1	SOD02	D	Unidentified	n/a	Female stands tail aside, male sniffs vulva	
1	Sodota	09/09/2007	4	SOD02	D	Unidentified	S	Mating and tied	An adult male approached the mating male and whilst he was tied with the female and behaved aggressively towards him, biting him
1	Sodota	16/09/2007	11	SOD02	D	Unidentified	n/a	Mating and tied	
1	Sodota	16/09/2007	11	SOD02	D	SOD07	S	Mounted, no tie	
1	Sodota	16/09/2007	11	SOD02	D	SOD01	D	Mounted, no tie	
1	Sodota	19/09/2009	14	SOD02	D	SOD01	D	Mounted, no tie	
1	Darkeena	05/09/2007	0	DAR02	D	Unidentified	n/a	Mating and tied	
1	Darkeena	05/09/2007	0	DAR02	D	Unidentified	n/a	Mating and tied	Mating male finally chased away by the Darkeena dominant male HAR15
1	Darkeena	05/09/2007	0	DAR02	D	DAR05	S	Mounted, no tie	DAR05 chased away from DAR02 by HAR15
1	Darkeena	05/09/2007	0	DAR02	D	HAR15	D	Mounted, no tie	
1	Darkeena	09/09/2007	4	DAR02	D	DAR03	S	Mounted, no tie	
1	Darkeena	09/09/2007	4	DAR02	D	DAR05	S	Mounted, no tie	
1	Addaa	02/10/2007	0	SOD06	D	SOD07	D	Mating and tied	SOD06 and SOD07 became dominant in Addaa pack after splitting from Sodota pack
2	Darkeena	06/11/2008	-2	DAR02	D	DAR03	D	Mounted, no tie	DAR03 became dominant after the death of HAR15
2	Sodota	15/11/2008	15	SOD02	D	SOD01	D	Mating and tied	
2	Darkeena	04/12/2008	27	DAR12	S	DAR03	D	Female stands tail aside, male sniffs vulva	DAR02 close to DAR12 and DAR03 but does not interfere
3	Megity	17/09/2009	-5	MEG02	D	DAR05	From another pack	Mounted, no tie	DAR05 tries to mount MEG02, MEG02 not receptive and lies down to prevent mating. Finally she runs away from DAR05 and mates with two other males
3	Megity	17/09/2009	-5	MEG02	D	DAR05	From another pack	Mounted, no tie	DAR05 tries to mount MEG02, MEG02 not receptive and lies down to prevent mating. Finally she runs away from DAR05 and mates with two other males
3	Megity	17/09/2009	-5	MEG02	D	GEN07	From another pack	Mating and tied	
3	Megity	17/09/2009	-5	MEG02	D	Unidentified	n/a	Mating and tied	
3	Megity	18/09/2009	-4	MEG06	D	ALA07	D	Female stands tail aside, male sniffs vulva	MEG06 and ALA07 became dominant after the dispersal of MEG02 and MEG01
3	Megity	18/09/2009	-4	MEG06	D	ALA01	From another pack	Female stands tail aside, male sniffs vulva	
3	Megity	22/09/2009	0	MEG06	D	ALA07	D	Mounted, no tie	
3	Megity	22/09/2009	0	MEG06	D	ALA07	D	Mounted, no tie	
3	Megity	22/09/2009	0	MEG06	D	ALA07	D	Mounted, no tie	
3	Megity	22/09/2009	0	MEG06	D	ALA07	D	Mounted, no tie	
3	Megity	22/09/2009	0	MEG06	D	ALA07	D	Mating and tied	ALA05 and ALA09 standing nearby
3	Megity	22/09/2009	0	MEG06	D	ALA09	S	Mounted, no tie	ALA09 chased away from MEG06 by ALA07
3	Dumal	26/09/2009	-5	DUM02	D	DUM01	D	Mating and tied	
3	BBC	06/10/2009	6	BBC32	D	Unidentified	n/a	Mating and tied	
3	BBC	07/11/2009	47	BBC42	S	Unidentified	From another pack	Mounted, no tie	