Stress and reproduction in domestic cats (*Felis catus*) as a model for endangered felids

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Synopsis

Captive breeding programs are a vital component of the conservation strategies for felids, but these programs are often hindered by poor reproductive performance. Knowledge of reproductive biology is crucial to improving *in situ* and *ex situ* felid breeding programs. This thesis provided the first comprehensive systematic review of the literature available on the reproductive biology of the extant felid species. It was concluded that the high prevalence of teratospermia and highly variable oestrous cycles in felids contribute towards their poor reproductive performance in captivity. The captive environment has been linked to reduced ejaculate quality and ovarian quiescence in felids, but it is difficult to elucidate whether this is due to captivity-related stress (i.e., elevated glucocorticoid (GC) concentrations) or other factors associated with captivity. This thesis aimed to determine whether a simulated endocrine stress response (GC treatment) altered the testicular and ovarian function of felids using the domestic cat as a model species. While epididymal sperm motility was unaffected by GC treatments, the percentage of morphological abnormal sperm was higher in GC-treated cats than in control cats. This would likely have an adverse effect on fertility as morphologically abnormal sperm are rarely involved in the fertilisation process. Glucocorticoid treatments did not affect the ovarian response of cats in which follicular growth and development was stimulated by exogenous gonadotrophins. However, ooplasm and zona pellucida morphology was graded poorer in GC-treated animals than control animals. Whether this corresponds to a reduction in fertility is unclear as the fertilisation capabilities of oocytes were not assessed. It would be worth investigating whether GC administration affects the natural oestrous cycles of cats, as elevated GC concentrations associated with captivity have been linked to ovarian quiescence. However, this would require an accurate and minimally invasive (i.e., low stress) method for monitoring the ovarian cycles of domestic cats. Thus, this thesis investigated whether accelerometry and infrared thermography could be used to monitor the ovarian function of cats. It was found that accelerometry could be used to detect an increase in activity of cats following the induction of follicular growth with equine chorionic gonadotrophin (eCG). Infrared thermography also identified changes in perivulvar temperature (PVT) driven by follicular development and ovulation, with PVT increasing as follicular growth occurred and decreasing following ovulation. Both methods show promise; however, further investigation into the use of accelerometry and IR thermography for monitoring ovarian function is needed. In conclusion, the results of thesis indicate that GC have adverse effects on the testicular and ovarian function of domestic cats. Thus, there is an urgent need to further investigate the effects of captivity-related stress on the reproductive performance of non-domestic felids. Furthermore, this thesis assessed two promising non-invasive methods for monitoring the ovarian activity of cats, with the findings being highly applicable for the management and breeding of non-domestic felids in captivity.

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List of Abbreviations

ABP	Androgen-binding protein
AC	Adenylate cyclase
ACTH	Adrenocorticotrophic-releasing hormone
AES	Abiotic environmental stressors
AI	Artificial insemination
ALT	Altrenogest
APCI	Atmospheric pressure chemical ionisation
AR	Androgen receptor
ARC	Arcuate nucleus
ART	Assisted reproductive technologies
AV	Artificial vagina
AVPV	Anteroventral periventricular nucleus
CSS	Confinement-specific stressors
CH	Corpora haemorrhagica
CITES	Convention on International Trade in Endangered Species
CL	Corpora lutea
CNS	Central nervous system
CRH	Corticotrophin-releasing hormone
CRH-R	Corticotrophin-releasing hormone receptor
cAMP	Cyclic adenosine monophosphate
D	Direct
DAB	Diaminobenzidine
DD	Data deficient
ΔG	Change in acceleration from gravity
DMN	Dorsomedial nucleus
eCG	Equine chorionic gonadotrophin
E_2	Oestradiol
EE	Electroejaculation
EN	Endangered
EP	Epididymal sperm collection
FEM	Faecal oestradiol-17β metabolites
FGM	Faecal glucocorticoid metabolites
FPM	Faecal progesterone metabolites

Frag.	Fragmentation
FSH	Follicle stimulating hormone
FSH-R	Follicle stimulating hormone receptor
GC	Glucocorticoid
GD	Genetic diversity
GnIH	Gonadotrophin inhibitory hormone
GnRH	Gonadotrophin-releasing hormone
GnRH-R1	Gonadotrophin-releasing hormone receptor type 1
GPR-54	G-protein-coupled receptor-54
GR	Glucocorticoid receptor
GSI	Gonadosomatic index
hCG	Human chorionic gonadotrophin
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic-pituitary-gonadal
HPLC	High-pressure liquid chromatography
I	Indirect
IR	Infrared
IUCN	International Union for Conservation of Nature
kiSS	Kisspeptin
LC	Least concern
LH	Luteinising hormone
LH-R	Luteinising hormone receptor
LO	Laparoscopic oviductal
LU	Laparoscopic uterine
ME	Median eminence
MMA	Actical® Minimitter accelerometers
MS	Mass spectrometry
Ms2	Tandem mass spectrometry
ND	No data
NL	Normalisation level
NPLP	Non-pregnant luteal phases
NS	Non-significant $P>0.10$
NT	Near threatened
OD	Oocyte diameter
OPA	Overall physical activity
PAT	Perianal temperature

PBS	Phosphate buffered saline
PFC	Pre-frontal cortex
pFSH	Porcine follicle stimulating hormone
PKA	Protein kinase A
PLC	Phospholipase C
pLH	Porcine luteinising hormone
PLP	Pregnant luteal phase
POA	Preoptic area
POMC	Proopiomelanocortin
Prev.	Previously
PVN	Paraventricular nucleus
PVT	Perivulvar temperatures
PVT-GT	Perivulvar temperature relative to the gluteal temperature
PVT-PAT	Perivulvar temperature relative to the perianal temperature
SMI	Sperm motility index
SRM	Selected reaction monitoring
rTdT	Recombinant terminal deoxynucleotidyl transferase
TC	Transcervical
TD	Seminiferous tubule diameter
TΔG	Total change in acceleration from gravity
TH	Seminiferous tubule height
TIC	Total ion chromatogram
TOS	Total oocyte score
TUNEL	rTdT dUTP nick end labelling
UC	Urethral catheterisation
V	Vaginal
VU	Vulnerable
ZP	Zona pellucida
ZP1-4	Zona pellucida thickness measurements 1-4

Chapter 1

General introduction and thesis objectives

Chapter 1: General introduction and thesis aims

1.1 The felid family: Felidae

Felidae is a diverse family consisting of 38 extant species (Figure 1.1), with native representatives on all continents except Australia and Antarctica (Johnson and O'Brien, 1997; Slattery and O'Brien, 1998; Johnson *et al.*, 2006). Johnson *et al.* (2006) conducted an extensive genetic-based study into the origins and phylogeny of felids. The study confirmed earlier research suggesting eight distinct felid lineages (Domestic cat lineage, Leopard cat lineage, Puma lineage, Lynx lineage, Ocelot lineage, Caracal lineage, Bay cat lineage, and Panthera lineage), and placed several previously wrongly classified species (marbled cats (*Pardofelis mamorata*), servals (*Caracal serval*), Pallas' cats (*Otocolobus manul*), and rusty spotted cats (*Prionailurus rubiginosus*)) into their appropriate lineages (Johnson and O'Brien, 1997; Slattery and O'Brien, 1998; Johnson *et al.*, 2006). For the purpose of this thesis, the felid species and linages discussed are those described by Johnson *et al.* (2006; Figure 1.1).

Figure 1.1 The phylogenetic and geographic origins of the extant felid species (Johnson *et al.*, 2006). This phylogeny has been used to define the Felidae family and species throughout this thesis.

1.2 Conservation status of felids

Felidae is one of the most widely threatened and endangered animal families, with many species requiring extensive conservation management (Nowell and Jackson, 1996). According to the International Union for Conservation of Nature (IUCN) Red List of Threatened species (IUCN, 2020), 25 of the 37 non-domestic felids are endangered or threatened in at least part of their natural home range (Nowell and Jackson, 1996; Nowell, 2002; Swanson, 2003). Furthermore, all 37 non-domestic species are listed in either appendix one or two of the Convention on International Trade in Endangered Species (CITES) treaty (Table 1.1), which was developed to regulate and restrict the trade of endangered species (CITES, 2017).

Table 1.1 Description of the three Convention on International Trade in Endangered Species (CITES) appendices (CITES, 2020).

Appendix number	Description
Appendix 1	Species threatened with extinction and CITES prohibits international trade in specimens of these species except when the purpose of the import is not commercial (see Article III), for instance for scientific research.
Appendix 2	Lists species that are not necessarily threatened now with extinction but that may become so unless trade is closely controlled. International trade in specimens of Appendix-II species may be authorized by the granting of an export permit or re-export certificate. No import permit is necessary for these species under CITES (although a permit is needed in some countries that have taken stricter measures than CITES requires).
Appendix 3	A list of species included at the request of a Party that already regulates trade in the species and that needs the cooperation of other countries to prevent unsustainable or illegal exploitation. International trade in specimens of species listed in this Appendix is allowed only on presentation of the appropriate permits or certificates.

Nowell and Jackson (1996) developed a system to rank felid species according to their vulnerability to extinction (i.e., vulnerability assessment; Table 1.2). The assessment assumes that the number of habitats, geographic range, and body size of a species are all linked to the vulnerability to extinction. Species that are restricted to one or two habitat types (e.g., black-footed cats (*Felis nirgripes*), sand cat (*Felis margarita*), or Iberian lynx (*Lynx pardinus*)), are far more vulnerable to habitat loss and fragmentation than species that can inhabit a wide range of environments (e.g., leopard cat (*Prionailurus bengalensis*), bobcats (*Lynx rufus*), or puma (*Puma concolor*)). A species that exhibits a smaller geographic range is also more vulnerable to habitat destruction (Nowell and Jackson, 1996). Interestingly, body size appears to be positively correlated with vulnerability to extinction in felids (Nowell and Jackson, 1996), perhaps due to the increased human-animal conflict with larger felid species or the larger home ranges and territories of these species (Nowell and Jackson, 1996).

The vulnerability scores and rankings, population trends, IUCN status, and CITES information for each of the 37 non-domestic felid species are summarised in Table 1.3. Black-footed cats and Iberian lynx are the most vulnerable species, with an extreme risk of extinction and a vulnerability ranking of one. Chinese mountain cats (*Felis bieti*), fishing cats (*Prionailurus viverrinus*), flat-headed cats

(*Prionailurus planiceps*), cheetah (*Acinonyx jubatus*), kodkod/güiña (*Leopardus guigna*), African golden cats (*Caracal aurata*), bay cats (*Pardofelis badia*), tigers (*Panthera tigris*), and snow leopards (*Panthera uncia*) are of particular risk of extinction (vulnerability ranking of 2). It is alarming that 17 felid species are classed as threatened across their entire range, and 28 of the 37 non-domestic species are exhibiting global population decline (IUCN, 2020). The aetiology of the declining population trends of most felids is highly variable, although habitat loss/fragmentation, decreased prey densities, human-animal conflict and illegal trade appear to be particularly problematic.

Table 1.2 Vulnerability assessments for felids (Nowell and Jackson, 1996). Felid species are given a score between -2 and +1 for habitat association (number of habitat types inhabited), geographic range, and body size (left side of the Table). These scores are summed to provide the total vulnerability score, which is used to determine the species vulnerability ranking/risk of extinction (right side of the Table). Vulnerability scores range from 0 (most vulnerable) to 5c (least vulnerable).

Vulnerability Score	**-2**	**-1**	**0**	**+1**
Habitat association (# habitats types)	-	Narrow (2-6)	Intermediate (7-9)	Broad (10+)
Geographic range ($x10^6$ km^2)	Restricted (<1.5)	Small (1.6-4.0)	Medium (5.0-9.0)	Wide (10.0-35.0)
Body size (kg)	-	Large (35-135)	Medium (7-20)	Small (<6.5)

Total Vulnerability Score	**Vulnerability Ranking**
-4	0
-3	1
-2	2
-1	3
0	4
+1	5a
+2	5b
+3	5c

1.3 Major threats to felids

1.3.1 Habitat loss and fragmentation

Anthropogenic habitat degradation is one of the most significant threats currently facing felids (Fergus, 1991; Mizutani, 1999; Seidensticker *et al.*, 1999; Kramer-Schadt *et al.*, 2004; Ray *et al.*, 2005; Michalski *et al.*, 2006; Swanepoel *et al.*, 2013; Wolf and Ripple, 2017). More than 60% of non-domestic felids have experienced high to severe habitat loss (Table 1.3). The current geographic ranges of cheetah, lions (*Panthera leo*), leopards (*Panthera pardus*), tigers, snow leopards and clouded leopards (*Neofelis nebulosa*) are substantially smaller that their historic ranges (only 8.5%, 6.3%, 20.6%, 4.7%, 22.4% and 36.3% of their historic range, respectively; Wolf and Ripple, 2017).

Many felids can inhabit a range of different habitat types, but there are some habitat specialists (e.g., sand cat, black-footed cat, Pallas' cat, fishing cat, Flat-headed cat, *Leopardis* spp., Andean mountain cat (*Leopardis jacobita*), and marbled cat; Table 1.3). It is generally these habitat specialists that have suffered most severely from habitat loss (Nowell and Jackson, 1996). Indeed, seven of the eight species listed above are severely threatened by habitat loss and fragmentation (Table 1.3). However, the effects of habitat loss have also become a severe problem for many species with a broad range of habitat associations (Table 1.3).

Table 1.3 Vulnerability assessments (described in table 1.2), CITES appendices (described in table 1.1), and threats faced for each of the 38 felid species (Nowell and Jackson, 1996; Hunter and Barrett, 2011; CITES, 2020; IUCN, 2020). The International Union for Conservation of Nature (IUCN) statuses herein are as follows: data deficient (DD), least concern (LC), near threatened (NT), vulnerable (VU), endangered (EN). Other abbreviations: fragmentation (frag.), genetic diversity (GD) previously (prev.).

Lineage	Common Name (*Scientific name*)	Vulnerability assessment				Threats					Population trend	IUCN Status	CITES
		Habitat variability (No. main habitats [Total No. Hábitats])	Geographic Range (x 10^6 km)	Body weight (kg)	Vulner-ability Ranking	Habitat loss and/or frag.	Decreased prey density	Persecuted for human-animal conflict	Illegal & commercial trade or hunting	Other			
Domestic cat	**Domestic cat** (*Felis catus*)	-	-	-	-	-	-	-	-	-	-	-	-
	European wild cat (*Felis silvestris*)	Broad *8 [12]*	Wide *34.17*	Small *3.5*	5c	Low	Moderate	Low	Low	Hybridisation Road mortality	Decrease	LC	A2
	African wild cat (*Felis libyca*)	Intermediate *6 [8]*	Wide *16.80*	Small -	5b	DD	DD	Low	DD	Hybridisation	DD	DD	A2
	Chinese mountain cat (*Felis bieti*)	Narrow *2 [5]*	Restricted *0.29*	Small *6.0*	2	DD	High	No conflict	Low	-	Decrease	VU	A2
	Desert/Sand cat (*Felis margarita*)	Narrow *2 [3]*	Medium *5.40*	Small *2.5*	4	Moderate	Moderate	Low	Low	Diseases from cats and dogs	DD	LC	A2
	Black-footed cat (*Felis nigripes*)	Narrow *3 [3]*	Restricted *0.95*	Small *1.2*	1	High	DD	No conflict	DD	By-kill from poison.	Decrease	VU	A1
	Jungle cat (*Felis chaus*)	Broad *8 [13]*	Medium *8.49*	Small *5.4*	5b	Moderate	Low	Low	Low	-	Decrease	LC	A2
Leopard cat	**Pallas' cat** (*Otocolobus manul*)	Narrow *4 [6]*	Medium *5.08*	Small *3.0*	4	High	Severe	No conflict	Moderate	-	Decrease	LC	A2
	Rusty spotted cat (*Prionailurus rubiginosus*)	Intermediate *7 [7]*	Restricted *0.78*	Small *1.5*	3	High	-	Low	Low	-	Decrease	VU	A1/A2
	Asian spotted/leopard cat (*Prionailurus bengalensis*)	Broad *7 [12]*	Medium *8.66*	Small *2.4*	5b	Moderate	-	Moderate	High	Hybridisation (low)	Stable	LC	A1/A2
	Fishing cat (*Prionailurus viverrinus*)	Narrow *5 [6]*	Small *2.33*	Medium *6.8*	2	Severe	High	Low	Moderate	Water pollution/prey contamination	Decrease	VU	A2
	Flat-headed cat (*Prionailurus planiceps*)	Narrow *3 [3]*	Restricted *1.18*	Small *1.9*	2	High	Moderate	No conflict	Moderate	Water pollution/prey contamination	Decrease	EN	A1

Lineage	Common Name (*Scientific name*)	Vulnerability assessment				Threats					Population trend	IUCN Status	CITES
		Habitat variability (No. main habitats [Total No. Hábitats])	Geographic Range (x 10^6 km)	Body weight (kg)	Vulner-ability Ranking	Habitat loss and/or frag.	Decreased prey density	Persecuted for human-animal conflict	Illegal & commercial trade or hunting	Other			
Puma	**Puma/mountain lion** (*Puma concolor*)	Broad *8 [15]*	Wide *17.12*	Large *41.0*	5a	High	Moderate	High	Moderate	Road mortality	Decrease	LC	A1/A2
	Jaguarondi (*Puma yagouaroundi*)	Broad *6 [10]*	Wide *13.54*	Small *4.4*	5c	High	-	Moderate	Low	-	Decrease	LC	A1/A2
	Cheetah (*Acinonyx jubatus*)	Intermediate *4 [8]*	Medium *7.35*	Large *43.0*	2	High	High	Moderate	Moderate	Genetic homogeneity	Decrease	VU	A1
Lynx	**Iberian lynx** (*Lynx pardinus*)	Narrow *3 [3]*	Restricted *0.08*	Medium *9.3*	1	Severe	Severe	Low	Moderate	Road mortality Genetic drift due to frag.	Increase	EN	A1
	Eurasian lynx (*Lynx lynx*)	Broad *6 [12]*	Wide *13.56*	Medium *17.0*	5b	Moderate	Moderate	High	High	Road mortality	Stable	LC	A2
	Canadian lynx (*Lynx canadensis*)	Intermediate *4 [8]*	Medium *5.06*	Medium *8.5*	4	Moderate	Moderate	No	Moderate	Competition with coyote	Stable	LC	A2
	Bobcat (*Lynx rufus*)	Broad *7 [11]*	Medium *7.24*	Medium *7.5*	5a	Moderate (frag.)	-	Low	High	Road mortality	Stable	LC	A2
Ocelot	**Ocelot** (*Leopardus pardalis*)	Intermediate *5 [9]*	Wide *12.45*	Medium *8.8*	5a	Moderate	-	Low	Moderate	Road mortality	Decrease	LC	A1
	Margay (*Leopardus wiedii*)	Narrow *2 [5]*	Medium *6.06*	Small *3.2*	4	Severe	-	Moderate	High (prev. severe)	Road mortality	Decrease	NT	A1
	Andean mountain cat (*Leopardus jacobita*)	Narrow *2 [2]*	Restricted *0.62*	Small *4.0*	2	Low	High	Low	Severe	-	Decrease	EN	A1
	Pampas cat (*Leopardus pajeros/colocolo*)	Broad *4 [10]*	Small *3.86*	Small *3.4*	5a	High	-	Moderate	low	By-kill from poisons High road mortality	Decrease	NT	A2
	Geoffroy's cat (*Oncifelis geoffroyi*)	Intermediate *6 [7]*	Small *2.80*	Small *4.2*	4	Moderate	-	Moderate	Low (prev. high)	-	Stable	LC	A1
	Güiña/Kodkod (*Leopardus guigna*)	Narrow *2 [4]*	Restricted *0.16*	Small *2.2*	2	Severe	-	Moderate to high	-	-	Decrease	VU	A2
	Tigrina/Oncilla (*Leopardus tigrinus*)	Narrow *3 [4]*	Small *2.90*	Small *2.0*	3	Severe	-	Low	Low (prev. high)	Road mortality	Decrease	VU	A1

Lineage	Common Name (*Scientific name*)	Vulnerability assessment				Threats					Population trend	IUCN Status	CITES
		Habitat variability (No. main habitats [Total No. Hábitats])	Geographic Range (x 10^6 km)	Body weight (kg)	Vulner-ability Ranking	Habitat loss and/or frag.	Decreased prey density	Persecuted for human-animal conflict	Illegal & commercial trade or hunting	Other			
Caracal	**Caracal** (*Caracal caracal*)	Intermediate *6 [10]*	Wide *18.99*	Medium *10.0*	5b (A)	High	-	Moderate	-	-	DD	LC	A1
	African golden cat (*Profelis/Caracal aurata*)	Narrow *2 [5]*	Small *2.46*	Medium *10.0*	2 (A)	Severe	Moderate	Low	Low	-	Decrease	VU	A2
	Serval (*Leptailurus/Caracal serval*)	Intermediate *7 [9]*	Medium *8.18*	Medium *10.0*	3	High	Moderate	Low	Moderate	-	Stable	LC	A2
Bay cat	**Bay cat** (*Pardofelis badia*)	Narrow *2 [2]*	Restricted *0.05*	Small *2.4*	2	Severe	DD	DD	Low	-	Decrease	EN	A2
	Timminck's/Asiatic golden cat (*Pardofelis temminckii*)	Intermediate *5 [8]*	Small *2.66*	Medium *10.0*	3	High	Low	Low	Moderate	-	Decrease	NT	A1
	Marbled cat (*Pardofelis marmorata*)	Narrow *3 [4]*	Small *2.42*	Small *3.5*	3	Severe	-	Low	High	-	Decrease	NT	A1
Panthera	**Lion** (*Panthera leo*)	Intermediate *5 [7]*	Medium *7.18*	Large *126.0*	3 (A)	Moderate (frag.)	High	Severe	High	Low GD in some populations	Decrease	VU	A1/A2
	Jaguar (*Panthera onca*)	Intermediate *4 [7]*	Medium *8.91*	Large *56.0*	3 (A)	High	High	High	Moderate	-	Decrease	NT	A1
	Leopard (*Panthera pardus*)	Broad *5 [15]*	Wide *23.14*	Large *40.0*	4 (A)	Moderate	High	Severe	High	-	Decrease	VU	A1
	Tigers (*Panthera tigris*)	Intermediate *6 [9]*	Small *1.99*	Large *136.0*	2 (A)	Severe	High	Severe	Severe	Reduced GD due to habitat frag.	Decrease	EN	A1
	Snow leopard (*Panthera uncial*)	Intermediate *1 [7]*	Small *2.39*	Large *37.5*	2 (A)	Moderate	High	High	High	-	Decrease	VU	A1
	Clouded leopards (*Neofelis nebulosa*)	Intermediate *4 [8]*	Small *2.79*	Medium *20.0*	3 (A?)	Severe	High	High	High	-	Decrease	VU	A1

The adverse effects of habitat loss and fragmentation on felids are exacerbated by the naturally large home ranges and territories of most species. Some of larger felids such as puma, cheetah, Eurasian lynx, and snow leopards have enormous home ranges (25-1000 km^2, 185-1850 km^2, 98-1850 km^2, and 58-4500 km^2, respectively; Sunquist and Sunquist, 2002; Kramer-Schadt *et al.*, 2004). Even many of the small and medium sized felids exhibit natural home ranges of 20-100 km^2 (Sunquist and Sunquist, 2002). These large natural home ranges are likely dictated by naturally low prey densities (Litvaitis *et al.*, 1986; Herfindal *et al.*, 2005; Schmidt, 2008; Loveridge *et al.*, 2009; Wolf and Ripple, 2016). Regardless of the causes, large home ranges ultimately mean that remnants of appropriate habitats are often too small to maintain effective population sizes of these species. Moreover, they are often too sparsely located to allow for regular dispersal and thus gene flow between populations (Palomares *et al.*, 2000; Kramer-Schadt *et al.*, 2004; Wilting *et al.*, 2006).

1.3.2 Poaching – legal and illegal hunting

Felids are frequently hunted for ingredients for traditional medicines, the pet trade, meat, pelts or other ornaments, and in retaliation for human-animal conflict (Nowell and Jackson, 1996; Hunter and Barrett, 2011). Despite numerous legislations restricting the hunting and trade of felids (Inskip and Zimmermann, 2009; CITES, 2020), hunting and trapping (both legal and illegal) remain a substantial threat to the existence of many species (Table 1.3; Nowell and Jackson, 1996). Furthermore, there is a strong association between risk of extinction and poaching intensity (Ferreras *et al.*, 1992; Kenney *et al.*, 1995; Palazy *et al.*, 2011; Li and Lu, 2014). The poaching of many felids has intensified over the past 30 years and is one of the main anthropogenic causes of mortality (Ferreras *et al.*, 1992; Kenney *et al.*, 1995; Haines *et al.*, 2005; Palazy *et al.*, 2011; Li and Lu, 2014). This is likely related to the increased threat status and reduced population numbers, since rarer species are of greater economic value as trophies (Palazy *et al.*, 2011). Palazy *et al.* (2011) called the positive association between threat status and poaching intensity the "cat dilemma", emphasising the significance of this association for felid conservation.

1.3.3 Decreased prey densities

The prey species of many felids have also been severely depleted due to anthropogenic habitat destruction, competition from domestic livestock, and hunting for resources (Nowell and Jackson, 1996; Damania *et al.*, 2003; Schmidt, 2008; Jumabay-Uulu *et al.*, 2014; Wolf and Ripple, 2016). More than a third of all felids have been at least moderately affected by reduced prey densities (Table 1.3), with low prey density being associated with starvation, high levels of intra-and inter-specific competition, and reduced reproductive performance (Nowell and Jackson, 1996; Wolf and Ripple, 2016). Most carnivores respond to decreasing prey density by increasing their home range (Wolf and Ripple, 2016). A negative correlation between home range size and prey density has been documented in felids, although the association is often weak as the home ranges of felids are highly variable and affected by various factors such as topography, sex, reproductive state, temporal or spatial variations

in prey density, and the amount of inter-intra-specific competition (Litvaitis *et al.*, 1986; Herfindal *et al.*, 2005; Schmidt, 2008; Loveridge *et al.*, 2009; Jumabay-Uulu *et al.*, 2014). Despite this, many felids have been shown to increase their natural home range in response to decreasing prey densities (Schmidt, 2008). Unfortunately, the destruction and fragmentation of suitable habitats has decreased the ability of felids to respond to reductions in prey density and forced many species to target alternative prey species (e.g., domestic animals).

1.3.4 Persecution due to human-animal conflict

Felids, especially larger species such as puma, Eurasian lynx (*Lynx lynx*), and *Panthera* spp., are often persecuted and killed by humans for preying on livestock or pets (Table 1.3). Felids have been reported to have killed a range of domestic animals (Patterson *et al.*, 2004; Bagchi and Mishra, 2006; Michalski *et al.*, 2006; Sangay and Vernes, 2008; Johansson *et al.*, 2015; Jędrzejewski *et al.*, 2017), but predation of livestock by lions, jaguars, leopards, tigers and snow leopards is of greatest economic consequence (Patterson *et al.*, 2004; Sangay and Vernes, 2008). The losses of livestock due to predation (by felids or other carnivores) imposes a significant financial burden on ranch owners (annual financial losses can be >20%; Inskip and Zimmermann, 2009). As a result, retaliation killing of felids by ranch owners is common, with many farmers believing that lethal control of felids is required regardless of their threat status or any financial compensation schemes (Bagchi and Mishra, 2006; Inskip and Zimmermann, 2009).

The attitude of people towards many *Panthera* spp. is worsened by the potential threat of these species to humans, since human attacks/killings by tigers, lions, leopards, and jaguars are also common (Inskip and Zimmermann, 2009). Tigers are responsible for more attacks on humans than all of the other *Panthera* spp. combined (Inskip and Zimmermann, 2009), but there are also reports of frequent attacks on humans by lions and leopards. Unsurprisingly, lions, leopards, and tigers inhabiting areas bordering human civilisation are frequently persecuted and killed due to the threat they pose to human lives, or in response to attacks.

1.3.5 Road mortality

Felid populations have been further fragmented by large road/highway networks, with many busy highways acting as barriers to migration and causes of mortality (Cain *et al.*, 2003; Kramer-Schadt *et al.*, 2004; Poessel *et al.*, 2014; Litvaitis *et al.*, 2015). High levels of road mortality have been reported for European wildcats, puma, and species within the lynx and ocelot lineages (Table 1.3). These species are possibly most prone to road mortality because their geographic range (i.e., part or all of their range in Europe or North America), is bisected by dense road networks and high traffic rates. The fragmentation of felid populations by roads is also problematic as it can lead to inbreeding, and thus reduced fertility (Obrien *et al.*, 1985; Menotti-Raymond and O'Brien, 1993; Pukazhenthi *et al.*, 2006b). In fact, extensive road networks have been a major contributor to the severe fragmentation of many felid species (Ferreras *et al.*, 1992; Ferreras, 2001). Felids generally have an aversion towards

open areas and prefer to construct territories in areas with low road densities, but habitat loss and reduced prey densities have forced many species to cross large roads and establish territories in areas with higher road densities (Maehr *et al.*, 1991; Taylor *et al.*, 2002; Cain *et al.*, 2003; Riley *et al.*, 2003; Haines *et al.*, 2005; Poessel *et al.*, 2014; Litvaitis *et al.*, 2015).

1.4 The importance of captive breeding programs for felid conservation

The major threats (e.g., habitat loss and poaching) faced by many felids have made *in situ* conservation programs extremely difficult. Even though translocation (to maintain genetic diversity in fragmented populations) and other conservation efforts (e.g., habitat protection and reconstruction) have been successful, they are often hindered by high non-natural mortality rates (poaching or road mortality; Nowell and Jackson, 1996; Inskip and Zimmermann, 2009; Palazy *et al.*, 2011; Poessel *et al.*, 2014; Wolf and Ripple, 2016; 2017). Captive breeding programs have thus become pivotal to the conservation of many felid species. However, the conservation value of captive breeding programs for aiding the recovery of felids is questionable, since captive animals are rarely released into the wild, and even if this occurs, post-release survival rates are low due to a number of factors including poor hunting success (Jule *et al.*, 2008).

The maintenance of felids in captivity does, however, provide an opportunity for researchers to investigate felid behaviour and physiology, the findings of which can be applied to improve both *ex situ* and *in situ* conservation efforts. For example, over the past decade there has been an increase in research effort towards the use of assisted reproductive technologies (ART) for felid conservation (Rodrigues da Paz *et al.*, 2005; Swanson, 2006; Rodrigues da Paz, 2012). This has largely been driven by the need to maintain or increase the genetic diversity of wild felid populations, although virtually all studies attempting artificial insemination (AI) on felids have been conducted on captive animals. Successful AI protocols would enable the transfer of genes between captive institutions or fragmented wild populations without the need for translocating animals, which is not only logistically challenging, but stressful for the animals. This would greatly enhance the conservation value of captive breeding programs for felids, and many other endangered species. At present, however, the use of ART in non-domestic felids has been limited by low success rates (Table 1.4; Barone *et al.*, 1994a; Howard *et al.*, 1996; Howard *et al.*, 1997; Roth *et al.*, 1997a; Pelican *et al.*, 2006; Thongphakdee *et al.*, 2020). In fact, even the 'natural breeding' of captive felids is often hindered by poor reproductive success, which suggests that captivity adversely affects felid reproduction (Mellen, 1991; Moreira *et al.*, 2007; Fanson *et al.*, 2010; Brown, 2011).

Table 1.4 Pregnancy rates of several felids following the artificial insemination (AI) with fresh or frozen sperm following natural or induced oestrus and induced ovulation. Table modified from Thongphakdee *et al.* (2020). Abbreviations: equine chorionic gonadotrophin (eCG), gonadotrophin-releasing hormone (GnRH), human chorionic gonadotrophin (hCG), laparoscopic uterine (LU), laparoscopic oviductal (LO), porcine follicle stimulating hormone (pFSH), porcine luteinising hormone (pLH), transcervical (TC), vaginal (V).

Species	Oestrus/ovulation induction	Sperm type	AI sperm deposition site	Pregnancy success (%)	Reference
Pallas cat	eCG/pLH	Fresh	LO	1 pregnant	(Swanson, 2012)
Leopard cat	eCG/hCG	Fresh/frozen	LU	25 (1/4)	(Wildt *et al.*, 1992)
	eCG/hCG	Fresh	LU	50 (1/2)	(Tajima *et al.*, 2016)
Puma	eCG/hCG	Fresh	LU	13 (1/8)	(Barone *et al.*, 1994b)
Cheetah	pFSH/hCG	Fresh/frozen	TC	0 (0/23)	(Wildt *et al.*, 1986)
	eCG/hCG	Fresh	LU	46 (6/13)	(Howard *et al.*, 1997)
	eCG/hCG	Fresh	LU	27 (3/11)	(Howard *et al.*, 1997; Howard *et al.*, 2002)
Ocelot	eCG/hCG	Fresh	LU	25 (1/4)	(Moraes *et al.*, 1997)
	eCG/hCG	Frozen	LU	25 (1/4)	(Moraes *et al.*, 1997)
	eCG/pLH	Fresh	LO	1 pregnant	(Swanson, 2012)
Tigrina	eCG/hCG	Fresh	LU	25 (1/4)	(Moraes *et al.*, 1997)
Lion	Natural/GnRH	Fresh	V/TC	29 (4/14)	(Callealta *et al.*, 2019)
Leopard	Natural/hCG	Fresh	TC	100 (1/1)	(Dresser *et al.*, 1982)
Snow leopard	eCG/hCG	Fresh	LU	7 (1/15)	(Roth *et al.*, 1997b)
Clouded leopard	eCG/hCG	Fresh	LU	5 (1/20)	(Howard *et al.*, 1997; Howard *et al.*, 2002)
	eCG/pLH	Fresh	LO	25 (1/4)	(Tipkantha *et al.*, 2017)
	eCG/pLH	Frozen	LO	20 (1/5)	(Howard *et al.*, 1996)

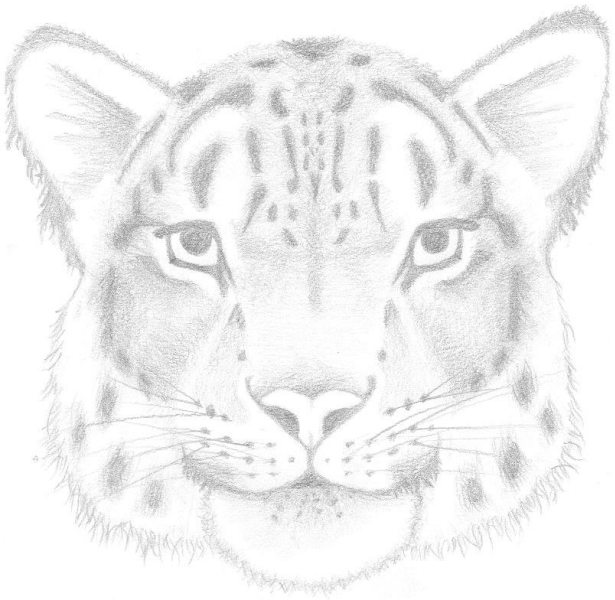
Unsurprisingly, the poor reproductive performance of many felids in captivity has been detrimental to their *ex situ* conservation. Captive populations of some felids, such as the cheetah, are not self-sustaining, with the annual death rates exceeding birth rates (Marker, 2012). Furthermore, only a small subset of captive felid populations are typically involved in captive breeding programs, greatly limiting the potential for maintaining and improving genetic diversity. As of 2013, only 20% of captive cheetahs in North America have reproduced (Grisham *et al.*, 2013). Breeding a greater number of captive individuals is often restricted by the need for transporting animals, and associated risks with intra-specific aggression and mate incompatibility (Foreman, 1997; Wielebnowski and Brown, 1998; Moreira *et al*., 2001; Brown *et al*., 2002; Wielebnowski *et al.*, 2002a; Henriksen *et al*., 2005; DeCaluwe *et al*., 2013; Thongphakdee *et al.*, 2018; Andrews *et al*., 2020; Thongphakdee *et al*., 2020). While the natural breeding is optimal for sustaining captive populations of felids, the aforementioned challenges, along with the poor fertility of many individuals, make this difficult (Thongphakdee *et al.*, 2018; Thongphakdee *et al.*, 2020). Consequently, improving the success of ART for felids is likely pivotal to the *ex situ* of felids (Thongphakdee *et al.*, 2018; Thongphakdee *et al*., 2020). The exact aetiology of the poor reproductive success and low pregnancy rates following ART procedures is unclear and likely to be multifactorial, but research into different aspects of the problem is important to improve the success of felid captive breeding programs.

1.5 Thesis aims

The ultimate aim of this thesis was to identify and assess some key factors that influence the reproductive performance of felids in captivity. This first required a detailed understanding of the normal reproductive biology of felids and how their reproductive performances differ between the wild and captivity. At the outset of this thesis, no comprehensive review of felid reproduction existed, so rectifying this became the first objective (Chapter 2). All other objectives were then derived from the detailed review (Chapter 2) and are thus presented at the end of Chapter 2 (section 2.8).

Chapter 2

Literature review and thesis objectives



Drawing by Farran McLean

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Andrews, C.J., Thomas, D.G., Welch, M.V., Yapura, J., Potter, M.A., 2020. Monitoring ovarian function and detecting pregnancy in felids: a review. *Theriogenology* 157, 245-253. A published version of this paper is available in Appendix 2**.**

Chapter 2: Literature review and thesis aims

2.1 Introduction

Felidae consists of 38 extant species that inhabit a wide variety of environments (Nowell and Jackson, 1996; Johnson *et al.*, 2006; IUCN, 2020). Twenty-five species are considered endangered or threatened in at least part of their natural geographic range, and all 37 non-domestic species are listed in the Convention on International Trade in Endangered Species (CITES) treaty (Chapter 1; Nowell and Jackson, 1996; Nowell, 2002; Swanson, 2003; Johnson *et al.*, 2006; CITES, 2020). Poaching (either directly or of prey species), culling, and habitat destruction or fragmentation are the main reasons cited for the high proportion of threatened species within Felidae (See Table 1.3). The impacts of these factors are so severe that *in situ* conservation efforts are not sufficient to ensure the persistence of many species.

Captive breeding programs have become an important component of the conservation strategies for many felid species, although the conservation value of such programmes is often questioned. Captive carnivores are rarely released into the wild, and, even if they are released, post-release survival rates are low due to a number of factors including poor hunting success (Jule *et al.*, 2008). However, the maintenance of felids in captivity does provide opportunities for investigation of the behaviour and physiology, and findings can be applied to improve both *ex situ* and *in situ* conservation efforts. In fact, the vast majority of the published literature on the reproductive biology and physiology of felids has been conducted on captive animals. A major problem with captive breeding programmes has been the poor reproductive performance of many species in captivity (Mellen, 1991; Terio *et al.*, 2004; Brown, 2006; Moreira *et al.*, 2007; Fanson *et al.*, 2010; Brown, 2011). Factors leading to poor reproductive success, and whether wild populations are also affected by such factors, can only be determined by having a thorough understanding of the reproductive biology of felids (Brown, 2006; Thongphakdee *et al.*, 2018). Until now, however, there has been no comprehensive review of their reproductive biology.

This review summarises literature on the reproductive biology of all extant felid species and identifies knowledge gaps. The methods used to monitor the ovarian and testicular function of felids are reviewed, with an emphasis on the advantages and limitations of each technique. The major factors that appear to affect the reproductive biology and fertility of felids are identified, and the implications of these for captive management are discussed. This thesis primarily focuses on the effects of stress on the reproductive performance of felids. Thus, a comprehensive review of the hypothalamic-pituitary-adrenal (HPA) axis and the effects of stress on the mammalian hypothalamic-pituitary-gonadal (HPG) axis is also included.

2.2 The hypothalamic-pituitary-gonadal axis

While there are more upstream regulators of the HPG axis (e.g., gonadotrophin inhibitory hormone (GnIH) and kisspeptin (kiSS), gonadotrophin-releasing hormone (GnRH) is released from neurons within the hypothalamus and is thought to be the master regulator of the HPG axis, and hence, gonadal function and activity (Merchenthaler *et al.*, 1984). Most vertebrates express multiple variants of GnRH, although GnRH1 is the most important regulator of the HPG axis in mammals (Naor, 2009). The perikarya of GnRH1 neurons are primarily located in the preoptic area (POA), medial septum, suprachiasmatic nucleus and arcuate nucleus (ARC) of the hypothalamus, and these neurons project into the median eminence (ME) and secrete GnRH1 into the hypophyseal-portal blood (Merchenthaler *et al.*, 1984).

There are three key GnRH receptors, but GnRH receptor type 1 (GnRH-R1) has been recognised as the most important for the HPG axis as it is highly expressed on gonadotroph cells of the anterior pituitary (Naor, 2009). Gonadotrophin-releasing hormone receptor type 1 is a $G_{q/11}$ protein coupled receptor that has an intracellular signalling pathway that involves phospholipase C (PLC; Naor, 2009). The adenylate cyclase (AC)-cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) pathway is also triggered directly by the binding of GnRH to GnRH-R1, since GnRH-R1 is also coupled to a G_{α} protein (Naor, 2009). Both the PLC and AC-cAMP-PKA pathways act to upregulate the synthesis and secretion of LH and FSH (Naor, 2009; Tsutsui *et al.*, 2010).

In males, LH binds to the LH-receptor (LH-R) to activate the AC-cAMP-PKA signalling pathway that ultimately results in the synthesis of the steroidogenic enzymes involved in testosterone production (Whirledge and Cidlowski, 2010). A similar response to LH is observed in the thecal cells of the ovary, where LH signalling promotes the production of androstenedione (from cholesterol), which is the precursor for oestradiol (Hillier *et al.*, 1994). Luteinising hormone has an additional role in the female, with the preovulatory LH surge inducing ovulation and promoting the formation of the corpus luteum (CL), and hence progesterone production; although, a number of other signalling molecules such as insulin-like growth factor 1 and transforming growth factor-β are also required (Richards *et al.*, 2002). The control of ovulation is outside of the scope of this thesis and will not be covered further in this chapter, suffice to say that high concentrations of LH are required for ovulation.

In the female, FSH binds to its G_s-coupled receptor (FSH-R) on the granulosa cells of the ovary to trigger AC, resulting in the production of cAMP and in turn, activation of PKA (Hillier *et al.*, 1994). PKA up-regulates the expression of LH-R, promotes follicular development and activates P450 aromatase, the enzyme required to convert thecal androstenedione/testosterone to oestrogens (Hillier *et al.*, 1994; Richards *et al.*, 2002). In the testis FSH stimulates Sertoli cells to promote spermatogenesis (Whirledge and Cidlowski, 2010). Ultimately, the gonadotrophins, LH and FSH,

stimulate steroidogenesis and gametogenesis in the male and female. A summary of the HPG axis and associated feedback loops can be seen diagrammatically in Figure 2.1.

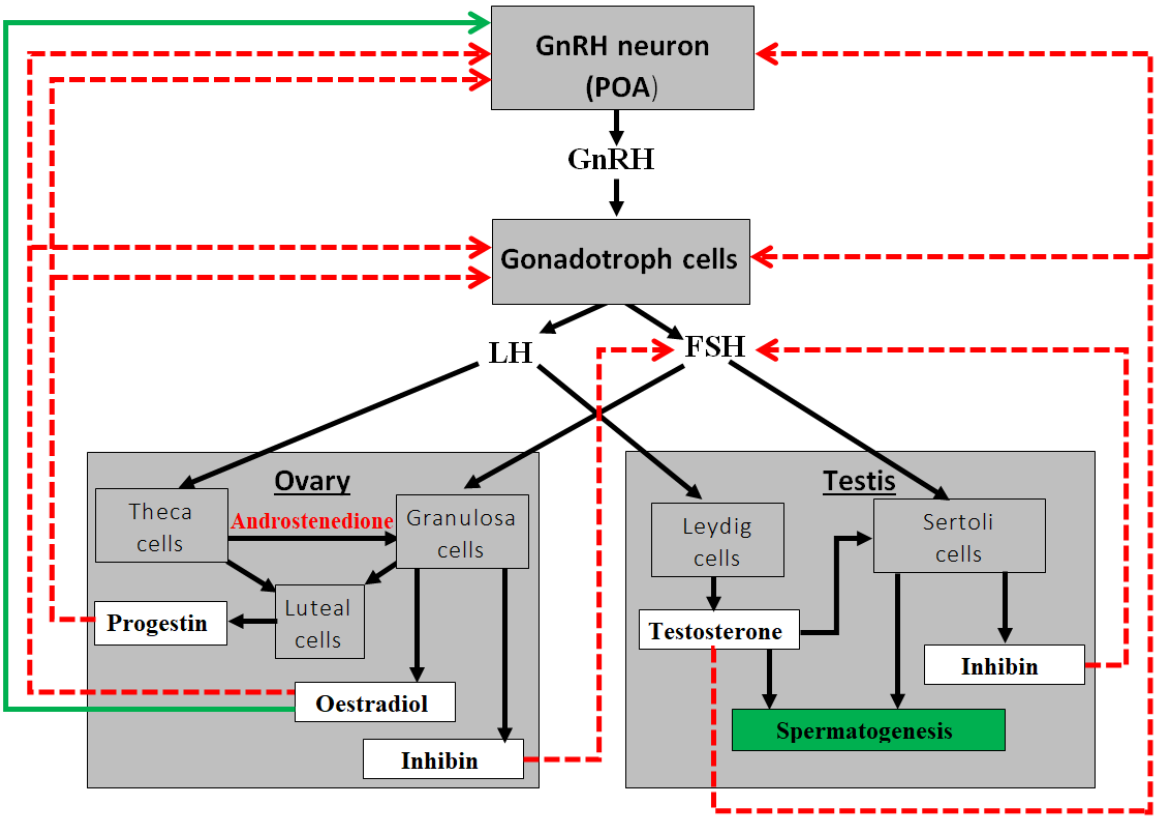

Figure 2.1 The hypothalamic-pituitary-gonadal (HPG) axis of male and female (red) animals with positive (solid green lines) and negative (dashed red lines) feedback loops. Note that the upstream regulators of gonadotrophin-releasing hormone (GnRH) such as gonadotrophin-inhibitor hormone (GnIH) and kisspeptin (kiSS) are not shown. Abbreviations: follicle stimulating hormone (FSH), gonadotrophin-releasing hormone (GnRH), luteinising hormone (LH), preoptic area (POA).

2.3 A review of the reproductive biology of felids

2.3.1 Methods used in literature selection

2.3.1.1 Literature review

Peer-reviewed literature and secondary papers (e.g., book chapters) on the reproductive biology of felids were reviewed for the period from 1941 to February 2021. Searches were conducted in both Web of Science and Google Scholar using the following terms:

Female felids - “Common name” OR “scientific name” OR “alternative name” AND breed* OR cycl* OR cytology OR estradiol OR estrogens OR estrous OR estrus OR felid* OR laparo* OR luteal OR ovar* OR pregnan* OR progest* OR prostagland* OR pseudopreg* OR reproduct* OR relaxin OR ultraso*.

Male felids - "Common name" OR "scientific name" OR "alternative name" AND andro* OR ejaculat* OR breed* OR electroejaculat* OR motil* OR morpholog* OR pleiomorphic OR reproduct* OR sperm* OR teratosperm* OR testosterone OR "uret* catheteri$ation".

The citation lists of all publications were checked for additional publications until no new relevant literature was discovered.

2.3.1.2 Summary statistics

The values reported are weighted means of the values presented in Appendix 1b and Appendix 1c, with 'n_E' representing the number of reproductive events [entire oestrous cycles, oestrus, interoestrus, non-pregnant luteal phases (NPLP; often referred to as pseudopregnancy), or ejaculates] and '*n*' being the number of individuals. Appendix 1b summaries the data available the oestrous cycle each of the extant felid species, all values were calculated as weighted means of the values reported in each of the publications cited, and are presented with a mean, range, and sample size (n, n_E). The ejaculate assessments from individual publications are presented in Appendix 1c, with values presented as means ± standard errors. The data from some publications in Appendix 1c were categorised according to collection method, sperm quality (teratospermic vs. normospermic males), season, habitat status (wild, captive on exhibit, or captive off exhibit), or genetic diversity (GD; i.e., males from populations with high or low GD), in order to determine whether and how these factors affect sperm quality in felids.

Statistical analyses were conducted using RStudio version 1.0.143 (R Foundation for Statistical Computing, Vienna, Austria) and a significance level of $P<0.05$. Normality was tested using a Shapiro-Wilk test. Parametric data were analysed using either a two-sample t-test or an analysis of variance and Tukey's post-hoc test. A Kruskal-Wallis test or pairwise Wilcoxon rank sum test was used to analyse non-parametric data.

2.3.2 Literature analysis

A total of 223 papers on felids were used for section 2.3; 173 of these focussed on the reproductive biology of felids. The literature was heavily biased towards the domestic cat (*Felis catus*), cheetah, and the panthera lineage (Figure 2.2). Research on the reproductive biology of non-domestic felids in the bay cat, caracal and domestic cat lineages was restricted to a few studies (Figure 2.2), many of which had very small sample sizes. The small amount of literature on caracals (*Caracal caracal*) and servals (*Caracal serval*) was surprising (only six and three publications, respectively) given the numbers of both these species in captivity.

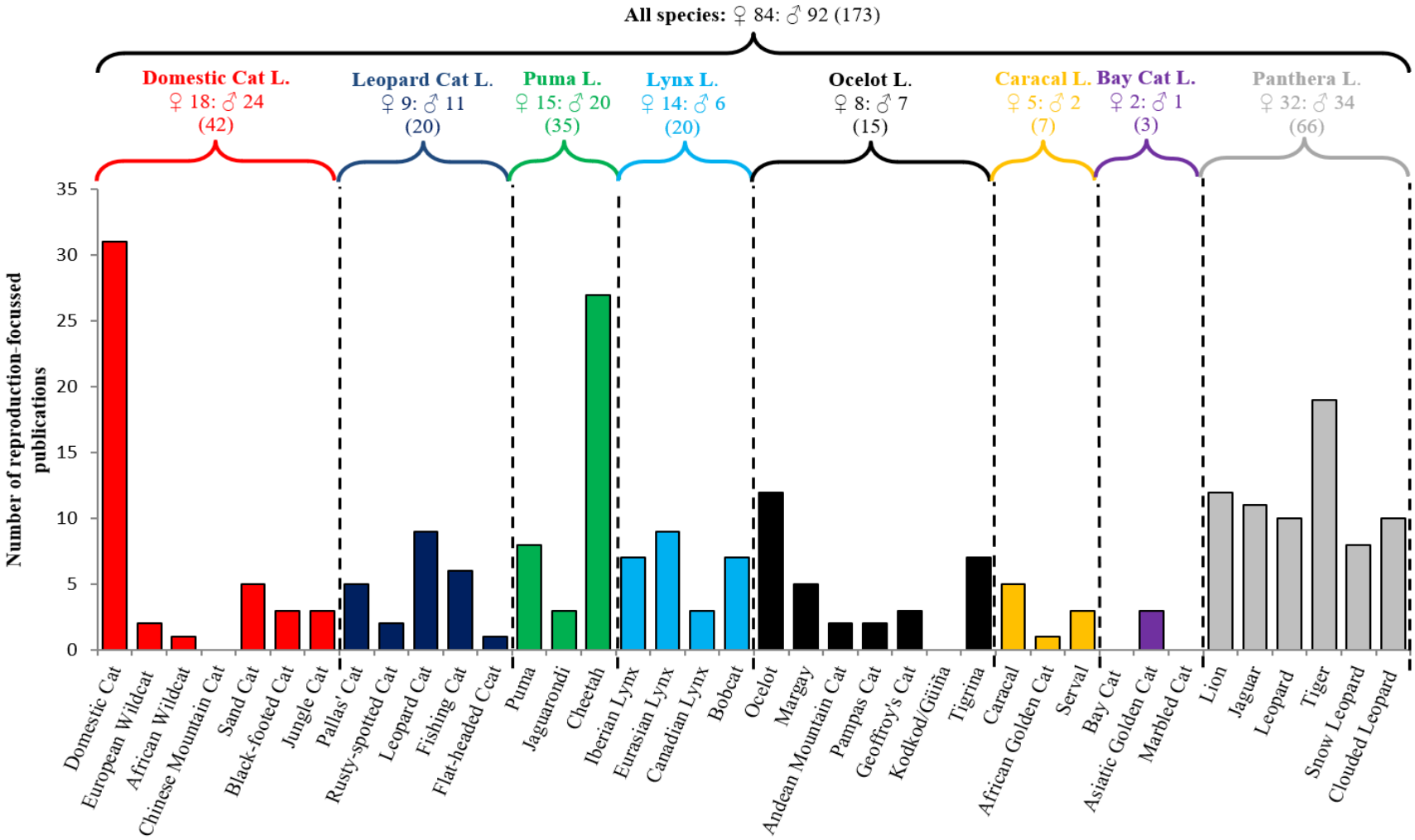

Figure 2.2 Numbers of publications on the reproductive biology of each of the 38 felid species, based on the publications listed in Appendix 1b and Appendix 1c. The total numbers of publications for each lineage (abbreviated as L.) do not necessarily equal the sum of the numbers of publications, because many publications included species from multiple lineages and/or information on the reproductive biology of both male and female felids. For the same reason, the total number of publications, number of female-focussed (♀) publications, and number of male-focussed (♂) publications for all species does not equal the sum of these values across lineages.

2.3.3 Reproductive biology of male felids

2.3.3.1 Methods used for semen collection

2.3.3.1.1 Electroejaculation

Electroejaculation (EE) was first used to collect ejaculates from domestic cats in the 1970s and has now become the most common method for sperm collection in felids (Platz and Seager, 1978; Wildt *et al.*, 1983; Wildt *et al.*, 1988; Morais *et al.*, 2002). The process of EE requires sedation and appears to have no detrimental effects on ejaculate quality, even if repeated more than once on a single animal (Platz and Seager, 1978). Electroejaculation requires a rectal probe (diameter ranging from 0.6 to 3.7 cm) with three longitudinal electrodes and an electrostimulator (Platz and Seager, 1978; Shivaji *et al.*, 1998; Pukazhenthi *et al.*, 2000; Crosier *et al.*, 2009; Erofeeva *et al.*, 2014). The probe is inserted into the rectum and the longitudinal electrodes positioned ventrally against the accessory sex organs (Shivaji *et al.*, 1998; Erofeeva *et al.*, 2014). The electrostimulator then sends a series of short (2-3 second) low voltage pulses to stimulate the nerves innervating the reproductive organs (Brown *et al.*, 1989; Shivaji *et al.*, 1998; Pukazhenthi *et al.*, 2000; Axnér and Linde-Forsberg, 2002). Semen is collected by placing the glans penis inside a pre-warmed Eppendorf tube (Axnér and Linde-Forsberg, 2002).

2.3.3.1.2 Artificial vagina

Ejaculates have also been collected from domestic cats using an artificial vagina (Sojka *et al.*, 1970; Axnér and Linde-Forsberg, 2002; Lambo *et al.*, 2012). The male is exposed to a "teaser female" (i.e., a female in oestrus) and allowed to mount without intromission (Sojka *et al.*, 1970; Lambo *et al.*, 2012). A rubber artificial vagina is placed over the glans penis and sperm is collected (Sojka *et al.*, 1970; Axnér and Linde-Forsberg, 2002; Lambo *et al.*, 2012). However, this method requires the male to be trained, and is only successful about 60% to 70% of the time (Platz *et al.*, 1978). Even in trained males, this technique is unlikely to be successful if the male is in unfamiliar surroundings, thus limiting the practical application of the technique (Axnér and Linde-Forsberg, 2002).

2.3.3.1.3 Urethral catheterisation

Semen has been collected successfully from domestic cats, jungle cats (*Felis chaus*), leopard cats (*Prionailurus bengalensis*), and lions (*Panthera leo*) using urethral catheterisation (UC; Lueders *et al.*, 2012; Lueders *et al.*, 2014; Cunto *et al.*, 2015; Kheirkhah *et al.*, 2017). The technique requires pharmacological induction to trigger the release of sperm into the urethra, which is most commonly achieved via an intramuscular injection of medetomidine (approximately 130 – 140 µg/kg of bodyweight; Zambelli *et al.*, 2008; Lueders *et al.*, 2012; Lueders *et al.*, 2014; Cunto *et al.*, 2015; Kheirkhah *et al.*, 2017). Ejaculates are collected via capilliary action into a catheter and pre-warmed Eppendorf tubes 20 – 40 minutes after pharmacological induction, depending on when

pharmacological effects are observed (Zambelli *et al.*, 2008; Lueders *et al.*, 2012; Lueders *et al.*, 2014; Cunto *et al.*, 2015; Kheirkhah *et al.*, 2017).

2.3.3.1.4 Epididymal sperm collection

Sperm have also been collected from the epididymis of domestic cats following castration or post-mortem (Neubauer *et al.*, 2004; Gañán *et al.*, 2009; Müller *et al.*, 2012; Gutiérrez-Reinoso and García-Herreros, 2016). The cauda epididymis is separated from the testis and immersed in a buffer medium (e.g., Hams 10 or phosphate buffered saline (PBS) buffer supplemented with pyruvate and/or foetal calf serum and an antibiotic), that has been warmed and maintained at 37ºC (Neubauer *et al.*, 2004; Müller *et al.*, 2012; Gutiérrez-Reinoso and García-Herreros, 2016). While immersed, the cauda epididymis is minced with a blade to release the sperm into the medium to create a "sperm suspension" that can be fixed for assessment or cryopreserved (Neubauer *et al.*, 2004; Gañán *et al.*, 2009; Müller *et al.*, 2012).

2.3.3.2 Assessment of felid sperm

2.3.3.2.1 Volume and concentration

The concentration of sperm within an ejaculate is evaluated using a haemocytometer/Neubauer chamber at 400 X magnification (Graham, 2001; Morato *et al.*, 2001; Pukazhenthi *et al.*, 2006a; Rodrigues da Paz, 2012). The overall concentration of sperm provides limited information about ejaculate quality, but it is needed to estimate the total number of viable, motile, and morphologically normal sperm. Both volume and concentration are greatly affected by the collection method used and are important consideration when extending and storing sperm for ART (e.g., dilution rates).

2.3.3.2.2 pH

The optimal ejaculate pH for felids appears to be between 6.5 and 9.0, though this differs slightly across species (Roth *et al.*, 1996; Axnér and Linde-Forsberg, 2002; Bertschinger *et al.*, 2008; Gañán *et al.*, 2010; Herrick *et al.*, 2010). An abnormally low pH indicates urine contamination, while a pH higher than the normal range suggests possible bacterial contamination (Rodrigues da Paz, 2012). A pH outside the physiologically 'normal' range can damage sperm cell integrity and decrease their motility, thus reducing the fertility of collected ejaculates (Roth *et al.*, 1996).

2.3.3.2.3 Sperm vitality/membrane integrity

The proportion of living sperm in an ejaculate is indicative of sperm quality and male fertility, since sperm must be alive to be fertile. Sperm vitality is generally determined through the use of two stains; a membrane permeable stain that stains living cells, and a non-permeable stain that only stains cells with compromised membrane integrity (i.e., dead cells). Staining combinations such as eosin/nigrosin or aniline blue and eosin/fast green FCF have been widely used to assess sperm vitality in felids via phase-contrast microscopy (van Dorsser and Strick, 2005; Thuwanut *et al.*, 2011). However,

fluorescent stains such as SYBR-14 and propidium iodide are often preferred as they provide improved contrast between living and dead sperm (Garner and Johnson, 1995; Graham, 2001). Flow cytometry can be used to automatically assess sperm vitality of ejaculate samples stained with fluorescent markers (Garner and Johnson, 1995; Graham, 2001), thus providing a more accurate and less labour-intensive assessment of sperm vitality.

2.3.3.2.4 Sperm motility

Sperm must also be motile in order to successfully locate and fertilise an oocyte (Graham, 2001). Sperm motility is generally determined by examining semen samples under a phase-contrast microscope and determining the percentage of motile sperm (Graham, 2001; Morais *et al.*, 2002). The percentage of motile sperm alone, however, provides limited information because it does not account for the type of movement exhibited by the sperm cells, which is a major factor influencing sperm transport. The type of movement exhibited by sperm can be assessed by subjectively scoring the sperm within a given sample according to a standardised progressive motility score (zero to five scale; Table 2.1; Howard, 1993; Morais *et al.*, 2002; Rodrigues da Paz, 2012). Together, the percentage of motile sperm and progressive sperm motility provide a good indicator of functional sperm motility in a given ejaculate. These two assessments are often combined to provide a standard sperm motility index (SMI) value, which is calculated as: SMI = (% motile sperm + (20* progressive motility))/2 (Morato *et al.*, 2001; Crosier *et al.*, 2009; Ganan *et al.*, 2009; Gañán *et al.*, 2010).

Table 2.1 Progressive motility scores and their characteristics (Howard, 1993).

Progressive motility score	Movement
0	No movement
1	Poor lateral movement with minimal linear movement
2	Moderate lateral movement with occasional linear movement
3	Slow linear movement
4	Linear movement
5	Rapid linear movement

2.3.3.2.5 Acrosome integrity

An intact acrosome is essential for the successful penetration of the zona pellucida (ZP) surrounding the oocyte by the sperm (Pukazhenthi *et al.*, 2006a). Various stains (e.g., Coomassie blue or fluorescently labelled plant lectins) can be used to assess acrosome integrity visually under a microscope, enabling researchers to classify sperm as having either a (1) normal intact, (2) abnormal intact, (3) normal non-intact or (4) abnormal non-intact acrosome (Gillan *et al.*, 2005; Pukazhenthi *et al.*, 2006a). In most studies, between 100 and 200 sperm cells are assessed to provide an estimate of the percentage of sperm with intact acrosomes within a given ejaculate sample (Crosier *et al.*, 2007; Pukazhenthi *et al.*, 2006a).

2.3.3.2.6 Sperm morphology

Morphologically abnormal sperm exhibit compromised acrosome reactions, impaired ZP binding, reduced metabolic activity (e.g., decreased lactate production), and reduced motility and are much less fertile than normal sperm (Howard, 1993; Pukazhenthi *et al.*, 2006b; Terrell *et al.*, 2010). Morphologically abnormal sperm are rarely involved in the process of fertilisation (Howard *et al.*, 1990; Howard, 1993; Long *et al.*, 1996; Pukazhenthi *et al.*, 2006b), and can even impair the function of morphologically normal sperm by compromising the tyrosine-kinase phosphorylation of proteins involved in the acrosome reaction and ZP binding (Howard, 1993; Long *et al.*, 1996; Pukazhenthi *et al.*, 1996; 1998).

Sperm morphology is generally assessed visually using light microscopy (1000 X magnification) with stains such as haematoxylin/eosin (Wildt *et al.*, 1988; Crosier *et al.*, 2007; Ganan *et al.*, 2009). An ejaculate sample is smeared on a slide and a defined number of sperm (normally between 100 to 300) are assessed (Wildt *et al.*, 1988; van Dorsser and Strick, 2005; Crosier *et al.*, 2007; Ganan *et al.*, 2009). A wide range of morphological abnormalities have been observed in felid ejaculates, and these abnormalities can be categorised as either primary or secondary in nature (Table 2.2; Wildt *et al.*, 1987b; Howard *et al.*, 1990; Crosier *et al.*, 2007).

Table 2.2 Types of morphological sperm abnormalities categorised as either primary or secondary abnormalities (Morato *et al.*, 2001; Morais *et al.*, 2002b; Crosier *et al.*, 2007).

Primary abnormalities	**Secondary abnormalities**
Macrocephalic	Detached head
Microcephalic	Detached flagellum
Polycephalic	Bent mid-piece with cytoplasmic droplet
Acrosomal defects	Bent mid-piece without cytoplasmic droplet
Abnormal mid-piece	Bent flagellum
Biflagellate	Proximal droplet
Tightly coiled flagellum	Distal droplet
Mitochondrial sheath aplasia	Spermatid

Primary abnormalities occur during spermatogenesis and are normally of genetic origin, either directly (e.g., impaired Sertoli cell function or number), or indirectly through genetically-linked andrological conditions (i.e., conditions resulting in abnormal testosterone concentrations; Pukazhenthi *et al.*, 2001; Chemes and Rawe, 2003; Pukazhenthi *et al.*, 2006b; Crosier *et al.*, 2009; Müller *et al.*, 2012; Jewgenow *et al.*, 2014). Secondary abnormalities are a consequence of abnormal conditions during sperm maturation and occur in the epididymis or ductus deferens. While secondary abnormalities are considered less severe than primary abnormalities, their aetiology is much more variable and difficult to identify (Pukazhenthi *et al.*, 2001; Morais *et al.*, 2002; Pukazhenthi *et al.*, 2006b). It is evident that secondary abnormalities are a consequence of the internal environmental conditions in which the sperm develop (e.g., hormone concentrations, seminal plasma composition and pH), but the factors that alter this internal environment can range anywhere from captivity-related

stress (stress induced decreased in testosterone concentrations) to dietary phytoestrogens (Setchell *et al.*, 1987).

2.3.3.3 Reproductive biology and ejaculate quality of male felids

Literature on ejaculate quality is available for 28 of the 38 felid species (Appendix 1c). No published research found on the ejaculate traits of European wild cats (*Felis silvestris*), African wild cats (*Felis lybica*), Chinese mountain cats, rusty-spotted cats, Andean mountain cats, kodkods, African golden cats, bay cats, or marbled cats. The methods used to collect ejaculates or spermatozoa from domestic cats have a considerable effect on sample volume ($P<0.05$) and sperm concentration ($P<0.05$; Figure 2.3). Methods other than EE have been used in only a few felid species (Appendix 1c), and comparable data are available only for domestic cats and lions. In the domestic cat, EE and epididymal sperm collections result in similar concentrations of sperm, while AV and UC provide lower sample volumes but substantially higher sperm concentrations (Figure 2.3). This is also apparent in the lion, where UC results in 'ejaculate' samples that are almost 80 times more concentrated than those obtained using EE [1940.0 x 10^6 sperm/mL (n_E=7) vs. 24.4 x 10^6 sperm/mL (n_E=48), respectively]. The significantly higher spermatozoa concentrations observed in 'ejaculates' collected by UC could be partly associated with the considerably smaller sample volumes obtained using this technique (Figure 2.3; Appendix 1c). The method of collection does not appear to have a significant effect on the number of abnormal sperm or on sperm motility, at least in the two species studied (i.e., lion and domestic cat).

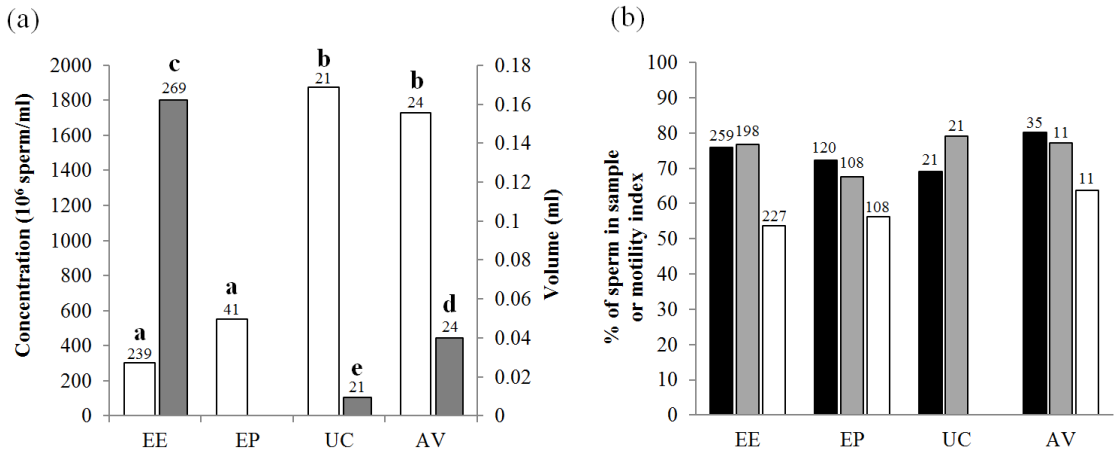

Figure 2.3 (a) Volume (grey) and sperm concentration (white) of ejaculates collected from domestic cats using four techniques: electroejaculation (EE), post-mortem epididymal sperm collection (EP), urethral catheterisation (UC), and artificial vagina (AV). Different letters indicate statistical significance ($P<0.05$), both within and between each variable. Sample sizes (i.e., number of 'ejaculates') are indicated as a number above each bar. Data on sample volume were not reported for epididymal collections. (b) Percentage of motile sperm (black), sperm motility index (SMI; grey) and percentage of morphologically abnormal sperm (white) in the ejaculates of domestic cats collected using EE, EP, UC, and AV. Sample sizes (i.e., number of ejaculates) are indicated as a number above each bar. Data on the percentage of abnormal sperm in samples collected using UC were not reported. The percentage of motile sperm, SMI, and percentage of morphologically abnormal sperm were similar between collection methods ($P>0.05$).

While SMI values differed between species (P<0.01), a Tukey's post hoc test revealed that pumas and tigers were the only species to have significantly different SMI values (lowest and highest mean SMI values of all species, respectively). In general, sperm motility is relatively high among felids (Appendix 1c), with a mean SMI across species of 69% (range: 26-90%, n_E=2104).

Sperm vitality (69%, range: 49-87%, n_E=443) and acrosome intactness (84%, range: 20-100%, n_E=1763) also appeared to be high in fresh ejaculates of most felids, although only 13 of the 92 studies that investigated felid ejaculate quality reported sperm vitality. In contrast, the percentage of morphologically abnormal sperm in felid ejaculates seemed high (61%, range: 3-98%, n_E=2882; Table 2.3), although there was considerable variation between the different felid lineages (P<0.001) and species (P<0.001). In fact, all publications from the puma and lynx lineages reported teratospermia (Appendix 1c; Table 2.3).

Table 2.3 Weighed means of the percentages of morphologically abnormal sperm in the ejaculates of species from each felid lineage. Values calculated from data in Appendix 1c. Abbreviation: no data (ND).

Lineage	n (ejaculates)	Total morphological abnormal sperm (%)			Primary abnormalities (%)	Secondary abnormalities (%)
		Weighted mean	Minimum	Maximum		
Domestic cat	473	53.0	23.8	90.7	7.7 (n_E=150)	30.1 (n_E=150)
Leopard cat	130	43.9	3.2	80.4	20.3 (n_E=40)	28.4 (n_E=40)
Puma	1055	75.6	63.2	93.5	34.5 (n_E=543)	44.6 (n_E=543)
Lynx	84	78.8	63.2	98.2	ND	ND
Ocelot	312	41.9	17.6	71.0	9.2 (n_E=157)	24.5 (n_E=157)
Caracal	7	29.4	12.0	36.4	ND	ND
Bay cat	1	61.0	ND	ND	ND	ND
Panthera	830	54.0	8.8	84.7	26.7 (n_E=506)	22.3 (n_E=506)
Overall mean	2882	60.6	3.2	98.2	25.5 (n_E=1396)	32.3 (n_E=1396)

Teratospermia is probably the most significant factor affecting the fertility of male felids and has been described multiple times in all felid lineages except for the caracal lineage, for which only two publications are available (Appendix 1c; Table 2.3). This is problematic given that morphologically abnormal sperm are rarely involved in the fertilisation process (Howard *et al.*, 1990; Long *et al.*, 1996; Pukazhenthi *et al.*, 2001; Pukazhenthi *et al.*, 2006b). However, it is difficult to elucidate the aetiology of teratospermia in felids, since both primary (26%, range: 3-63%, n_E=1396) and secondary (32%, range: 3-54%, n_E=1396) sperm abnormalities are highly prevalent (Table 2.3). While secondary abnormalities are more prevalent (P=0.028), there is considerable variability between species. It is possible that the high incidence of teratospermia is due, in part, to poor GD, as species with poor GD (e.g., cheetah and puma) exhibit much higher percentages of morphologically abnormal sperm (Appendix 1c; Table 2.3). Furthermore, lions from populations with low GD produce ejaculates with much higher (approximately double) percentages of abnormal sperm than individuals from populations with greater genetic variance (Wildt *et al.*, 1987b, Brown *et al.*, 1991).

An alternative cause for the high proportions of abnormal sperm in felid ejaculates is the captive environment itself (e.g., captivity-related stress or diet). The ejaculate traits of captive and wild individuals have only been compared in puma, cheetah, Iberian lynx, lions, and jaguars (*Panthera onca*; Table 2.4). Captive jaguars and lions appear to produce ejaculates with higher percentages of morphologically abnormal sperm than their wild counterparts, while the ejaculate quality of captive pumas, cheetah, and Iberian lynx do not appear to differ greatly from that of their wild conspecifics (Table 2.4). This inconsistency is possibly related to the small number of ejaculates that have been collected and assessed from wild felids. Alternatively, it also possible that captive populations exhibit poorer GD than wild populations, which in turn, could contribute to reduced sperm quality; however, further research is required.

Table 2.4 The weighted means of ejaculate characteristics of captive and wild pumas, cheetahs, Iberian lynxes, jaguars, and lions. Values calculated from data in Appendix 1c. Sperm motility index (SMI; %) = (% motile sperm + (20* progressive motility))/2. 'n' represents the number of ejaculates. Abbreviation: no data (ND).

Species	Lifestyle	n	Volume (mL)	Concentration (x 10^6/mL)	SMI (%)	Morphologically abnormal (%)
Puma	Captive	40	2.9	23.9	61.2	82.5
	Wild	47	2.7	12.8	56.5	80.9
Cheetah	Captive	831	1.5	30.3	51.1	74.0
	Wild	62	ND	26.7	68.2	80.3
Iberian lynx	Captive	20	0.4	15.4	70.2	73.0
	Wild	4	0.5	10.1	55.8	74.1
Jaguar	Captive	178	6.6	9.7	58.3	52.0
	Wild	7	4.1	35.0	72.0	26.5
Lion	Captive	32	2.3	14.7	61.0	56.6
	Wild	33	7.0	21.2	82.4	40.4
Totals	Captive	1110	2.4	26.0	53.4	70.0
	Wild	156	2.6	11.8	67.3	69.5

2.3.4 Reproductive biology of female felids

2.3.4.1 The generalised feline oestrous cycle

A detailed summary of the literature (84 publications) on the basic female reproductive biology and oestrous cycles of all 38 felid species is provided in Appendix 1b. The oestrous cycle of female felids consists of four phases: anoestrus, follicular phase (pro-oestrus and oestrus), interoestrus (while technically part of follicular phase, interoestrus has been considered separately within this review), and luteal phase/dioestrus (Figure 2.4; Brown, 2011).

2.3.4.1.1 The follicular phase: pro-oestrus and oestrus

The follicular phase of the oestrous cycle, which includes both pro-oestrus and oestrus, is defined by the presence of developing ovarian follicles (Chatdarong, 2003; Bristol-Gould and Woodruff, 2006). Pro-oestrus is relatively short in most felids (less than 24 h), and is marked by the presence of small, developing, primary or secondary follicles (Bristol-Gould and Woodruff, 2006; Brown, 2011). Developing follicles secrete oestradiol at an increasing rate, so pro-oestrus is also accompanied by a gradual rise in plasma oestradiol concentrations (Griffin, 2001; Chatdarong, 2003; Bristol-Gould and

Woodruff, 2006; Malandain *et al.*, 2011). The indirect positive feedback of oestradiol on hypothalamic GnRH neurons via hypothalamic kisspeptin neurons up-regulates the activity of the HPG axis to further stimulate ovarian folliculogenesis and steroidogenesis (Kauffman *et al.*, 2007; Smith *et al.*, 2007; Popa *et al.*, 2008). This positive feedback loop is responsible for the continued growth and development of follicles during the follicular phase.

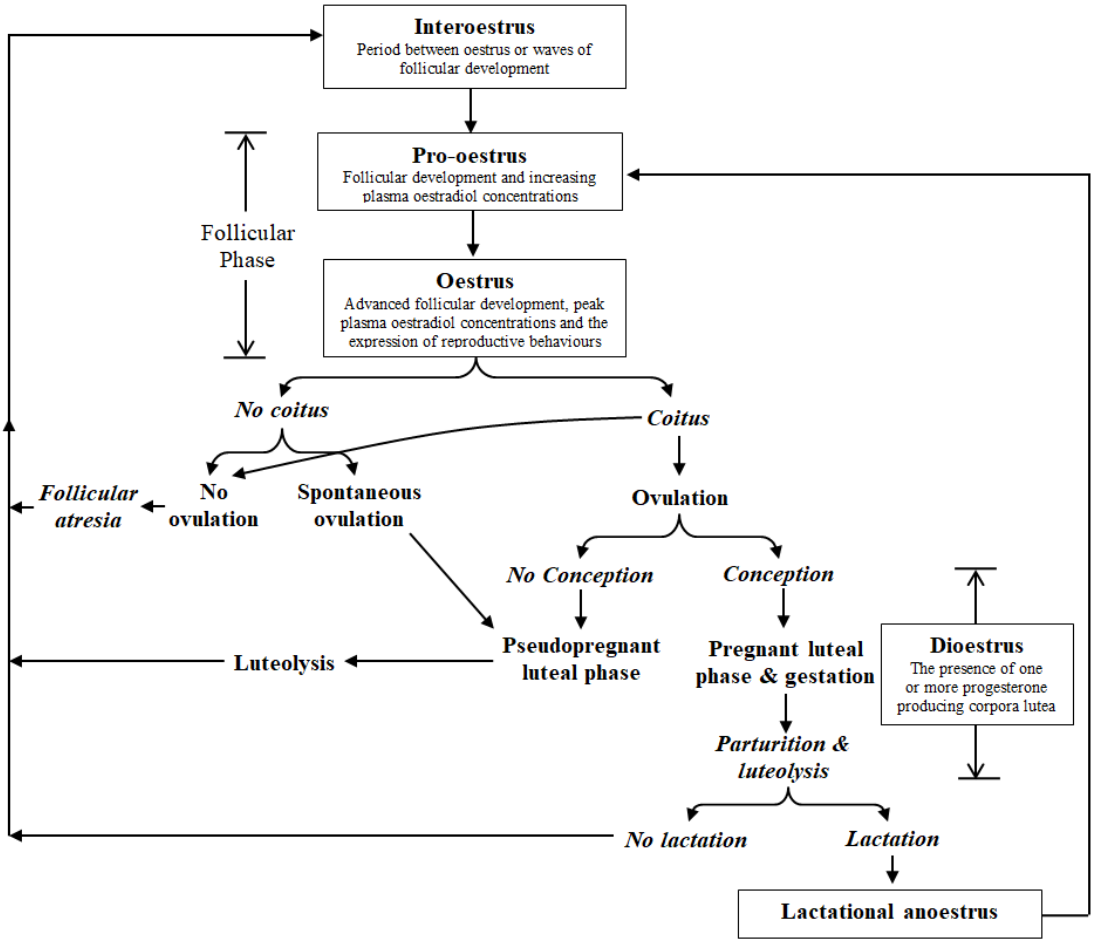

Figure 2.4 The generalised polyoestrous cycle of non-seasonal felids.

Endocrine oestrus is defined by the presence of one or more mature tertiary or dominant follicles, and peak oestradiol concentrations (Bristol-Gould and Woodruff, 2006; Brown, 2011; Malandain *et al.*, 2011). Oestradiol is the primary stimulant of reproductive behaviours in domestic cats, with exogenous oestradiol treatments triggering the expression of oestrous behaviours in ovariectomised domestic cats (Michael and Scott, 1964; Whalen and Hardy, 1970). In contrast to the need for progesterone priming in spontaneous ovulators, oestradiol appears to be the only reproductive hormone required to stimulate the expression of oestrous behaviours in induced ovulators such as felids (Michael and Scott, 1964; Whalen and Hardy, 1970; Bakker and Baum, 2000). Oestrous behaviours are relatively consistent among felid taxa, with most species exhibiting an increase in the

frequency of grooming, allogrooming, rubbing, rolling, scent-marking, locomotor activity, vocalisation or calling, and lordosis (Blomqvist and Sten, 1982; Mellen, 1993; Umapathy *et al.*, 2007; Kinoshita *et al.*, 2009; Brown, 2011). The intensity of behavioural oestrus, however, differs considerably within and between felid species, and oestrus signs range from overt and easily detectable to hidden and undetectable (Blomqvist and Sten, 1982; Asa *et al.*, 1992; Mellen, 1993; Foreman, 1997; Wielebnowski and Brown, 1998; Morato *et al.*, 2001; Brown *et al.*, 2002; Henriksen *et al.*, 2005; van Dorsser *et al.*, 2007; Kinoshita *et al.*, 2009; Siemieniuch *et al.*, 2012; Putman *et al.*, 2015). Felids are primarily induced or reflex ovulators, with coital stimuli required for the induction of ovulation. Coitus activates tactile neurons in the vagina and cervix, initiating an afferent signalling pathway that leads to the stimulation of hypothalamic GnRH neurons, and thus the pre-ovulatory GnRH/LH surge (Bakker and Baum, 2000; Richards *et al.*, 2002). In felids, the magnitude and duration of the pre-ovulatory GnRH/LH surge is dependent on the number and frequency of copulations, and multiple matings are typically required to induce ovulation (Concannon *et al.*, 1980; Shille *et al.*, 1983; Glover *et al.*, 1985; Schramm *et al.*, 1994; Foreman, 1997; Bakker and Baum, 2000).

2.3.4.1.2 Interoestrus

Most felids are polyoestrous, exhibiting multiple oestrous events throughout the year or breeding season (Graham *et al.*, 1995; Brown, 2011). If ovulation does not occur during oestrus, then there is a period of ovarian quiescence before another oestrous event. The period between consecutive oestrous events is referred to as interoestrus and is associated with basal oestradiol concentrations.

2.3.4.1.3 The luteal phase: dioestrus

If ovulation occurs, the remaining theca and granulosa cells of the ruptured follicles are transformed into CL, a process called luteinisation (Richards *et al.*, 2002; Feldman and Nelson, 2004). This phase of the oestrous cycle is referred to as dioestrus and is associated with a rise in plasma progesterone concentrations. Elevated progesterone concentrations have an important role in the maintenance of pregnancy in most species (Senger, 1997; Griffin, 2001).

If conception occurs following ovulation, then dioestrus will consist of a pregnant luteal phase (PLP). Progesterone concentrations generally remain elevated throughout gestation; however, temporary mid-gestational decreases in plasma progesterone concentrations have been observed in felids (Czekala *et al.*, 1994; Brown *et al.*, 1995; Graham *et al.*, 1995; van Dorsser *et al.*, 2007; Malandain *et al.*, 2011). The physiological significance of this drop in progesterone is not entirely clear, but it may reflect a switch from luteal to placental progesterone production (Briggs *et al.*, 1990; Brown *et al.*, 1995; Feldman and Nelson, 2004; van Dorsser *et al.*, 2007). Indeed, placental progesterone alone appears sufficient for the maintenance of pregnancy during late gestation, since a mid-gestational ovariectomy does not terminate pregnancy in domestic cats (Malassiné and Ferré, 1979; Brown *et al.*,

1995). Regardless of the source, progesterone concentrations recover quickly from any mid-gestational decreases and remain elevated through to parturition, returning to baseline levels just after birth (Czekala *et al.*, 1994; Graham *et al.*, 1995).

Felids also exhibit prolonged non-pregnant luteal phases (NPLP), which occur following spontaneous ovulations or sterile coitus-induced ovulations (Feldman & Nelson 2004, Brown 2011, Malandain *et al.*, 2011). Plasma progesterone concentrations observed during PLP and NPLP are similar, although NPLPs are shorter in duration (Appendix 1b; Brown *et al.*, 1995; Graham *et al.*, 1995; Dehnhard *et al.*, 2012).

2.3.4.1.4 Anoestrus

Anoestrus is a prolonged phase of reproductive acyclicity that is normally associated with the non-breeding season. Felids also exhibit a long period of reproductive inactivity following parturition and while young are suckling, referred to as lactational anoestrus (Feldman and Nelson, 2004; Brown, 2011).

2.3.4.2 Methods for monitoring the ovarian function of felids

Five main methods have been used to monitor the reproductive state of felids: behavioural-based assessments, endocrine (oestradiol and progesterone) monitoring, vaginal cytology, ultrasonography, and laparoscopy (Figure 2.5). Endocrine-based monitoring of reproductive state was the most commonly used method (110 publications across 26 species), followed by laparoscopy (70 publications across 19 species), and behavioural-based assessments (61 publications across 24 species; Figure 2.5). However, laparoscopy was predominantly used to determine the ovarian response to exogenous gonadotrophin treatments and to guide assisted reproductive techniques (ART). Vaginal cytology and ultrasonography were used relatively infrequently (7% and 13% of publications, respectively; Figure 2.5).

2.3.4.2.1 Behaviour

In many animals, oestrus can be detected by observing overt behavioural changes, although this requires a thorough understanding of the behaviour of each species. The behavioural changes associated with oestrus can also differ considerably between individuals of the same species or even within a single individual (Silva *et al.*, 2017). In addition, high levels of captivity-related stress can mask or inhibit behavioural changes that are normally linked to oestrus (Silva *et al.*, 2017).

In some felids, oestrus is associated with an expression of specific 'reproductive behaviours' (e.g., lordosis or calling/prusten; Blomqvist and Sten, 1982; Mellen, 1993; Graham *et al.*, 1995; Foreman, 1997; Umapathy *et al.*, 2007; Kinoshita *et al.*, 2009; Brown, 2011). For many species such as the domestic cat, clouded leopard, lion, snow leopard, and leopard, these 'oestrous' behaviours are overt and can often be used to indicate oestrus, although behaviourally silent oestrus events are also

commonly reported in many of these species (Blomqvist and Sten, 1982; van Dorsser *et al.*, 2007; Kinoshita *et al.*, 2009; Brown, 2011; Putman *et al.*, 2015). However, many other felids lack overt behavioural indicators of oestrus, for example, cheetah (Wildt *et al.*, 1981b; Asa *et al.*, 1992; Wielebnowski and Brown, 1998), *Leopardis* spp. (Moreira *et al.*, 2001), Eurasian lynx (Henriksen *et al.*, 2005), Pallas' cat (Brown *et al.*, 2002), and Geoffroy's cat (*Leopardus geoffroyi*; Foreman, 1997). Consequently, alternative methods for detecting oestrus in felids are required.

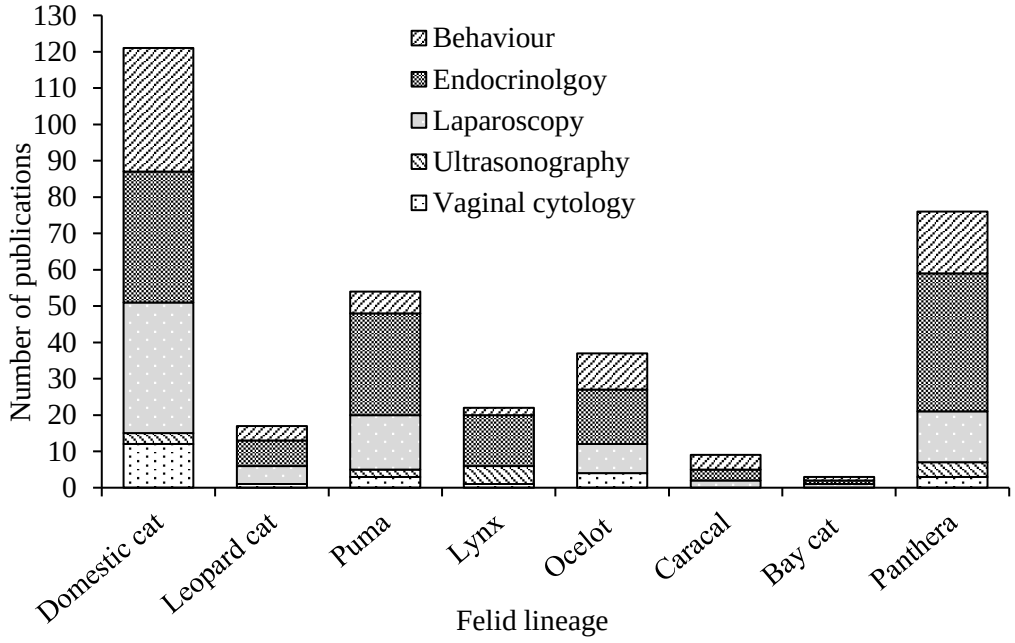

Figure 2.5 Number of publications that have used, or investigated the use of, various methods for monitoring ovarian activity of species within the eight felid lineages. Five main methods were used: behavioural-based assessments (behaviour), oestradiol and progesterone monitoring (endocrinology), laparoscopy, ultrasonography, and vaginal cytology. Note that some publications used these methods on multiple species, so are counted more than once.

2.3.4.2.2 Endocrine monitoring

Traditionally, blood hormone analyses are used to accurately monitor the reproductive state of animals, with elevated oestradiol concentrations being indicative of oestrus (i.e., follicular growth), and elevated progesterone concentrations associated with dioestrus (i.e., the presence of active corpora lutea and suppressed follicular growth; Bristol-Gould and Woodruff, 2006; Malandain *et al.*, 2011). Longitudinal assessment of circulating oestradiol and progesterone concentrations can thus be used to accurately monitor ovarian activity. Circulating oestradiol concentrations have been used to assess the ovarian activity of various felids, with oestrus being marked by oestradiol concentrations that are approximately 11 times higher than baseline (i.e., during anoestrus or interoestrus; Table 2.5; Appendix 2b; Andrews *et al.*, 2020).

However, the use of a single blood sample to determine reproductive state is not reliable, as reproductive steroid concentrations tend to be highly variable over time (Brown and Wildt, 1997). In addition, blood samples are normally collected using jugular venepuncture, an invasive and stressful procedure. While domestic cats can be conditioned to accept serial or repeated blood sampling, non-domestic felids often need to be anesthetised to obtain a blood sample. In the domestic cat, anaesthesia and/or stress have been found to alter the concentrations of reproductive hormones, disrupt ovarian cyclicity, impair sperm transport, and interfere with or even prevent ovulation (Howard *et al.*, 1992b; Brown and Wildt, 1997; Jurke *et al.*, 1997; Moreira *et al.*, 2007). Furthermore, anaesthesia can also increase the risk of foetal mortality (Howard *et al.*, 1992a; Wielebnowski and Watters, 2007; Braun *et al.*, 2009; Dehnhard *et al.*, 2012). Consequently, blood-based oestradiol analyses are rarely used to assess the ovarian activity of non-domestic felids, with >65% of studies using non-invasive faecal or urinary steroid metabolite assessments instead (Appendix 2b).

Table 2.5 A summary of the serum/plasma oestradiol (pg/mL) and faecal oestradiol metabolite (FEM; ng/g faeces) concentrations during anoestrus/interoestrus (basal) and oestrus in the eight felid lineages. Basal and luteal (i.e., peak) concentrations of serum/plasma progesterone (ng/mL) and faecal progesterone metabolites (FPM; µg/g faeces) are also shown. Values are presented as mean and range. Values <10 are presented with one decimal place. No data = ND. All data have been derived from Appendix 2b.

Lineage	Serum or plasma				Faecal metabolites			
	Oestradiol (pg/mL)		Progesterone (ng/mL)		FEM (ng/g)		FPM (µg/g)	
	Basal	Oestrus	Basal	Luteal	Basal	Oestrus	Basal	Luteal
Domestic cat	11 (1.1-20)	67 (21-160)	0.7 (<0.1-3.1)	40 (2.1-187)	592 (26-2617)	1524 (70-4279)	3.6 (0.1-11)	40 (5.0-135)
Leopard cat	ND	ND	ND	ND	174 (28-1335)	605 (149-910)	3.0 (1.0-16)	59 (13-141)
Puma	5.0 (1.0-30)	278 (30-430)	2.6 (<0.1-6.2)	~13	98 (2.3-86)	826 (27-1900)	0.2 (<0.1-8.0)	84 (3.4-1364)
Lynx	2.3 (<0.1-10)	ND	4.1 (0.9-7.2)	24 (0.8-168)	354 (282-446)	690 (542-878)	ND	8.2 (4.9-18)
Ocelot	10 (<0.1-32)	350 (74-586)	1.5 (0.5-2.9)	19 (8.9-12)	95 (10-200)	2175 (2500-8800)	8.9 (<0.1-30)	296 (35-1600)
Caracal	ND	ND	ND	ND	~10	24 (17-33)	0.3	2.5 (0.9-3.9)
Bay cat	ND	ND	ND	ND	~250	1800 (1200-2400)	ND	ND
Panthera	6.2 (0.2-23)	79 (19-440)	6.0 (<0.1-13)	44 (9.9-282)	268 (0.8-1400)	1060 (28-15980)	2.6 (0.3-25)	46 (0.5-585)
Average	8.7 (<0.1-32)	93 (19-586)	1.7 (<0.1-13)	38 (1.0-282)	260 (0.8-2617)	1042 (17-15980)	2.6 (<0.1-30)	55 (0.9-1600)
Fold increase from basal	-	11x	-	22x	-	4x	-	21x

In felids, oestradiol and progesterone metabolites are almost exclusively excreted through faeces (Shille *et al.*, 1984; Brown and Wildt, 1997). Faecal oestradiol-17β metabolites (FEM) and faecal progesterone metabolites (FPM) provide an alternative, indirect and non-invasive means of monitoring blood concentrations of these hormones (Brown and Wildt, 1997). In addition, faecal oestrogens and progestins tend to fluctuate less than circulating oestradiol and progesterone (Brown and Wildt, 1997).

Several techniques (e.g., radioimmunoassay, enzyme-linked immunosorbent assay, high-pressure liquid chromatography (HPLC), gas chromatography/mass spectrometry, and radiolabelling studies) have been validated and used to assess FEM and FPM concentrations, and thus monitor the reproductive status, of approximately 20 of the 38 felid species (Andrews *et al.*, 2020). There is considerable variation in the FEM and FPM concentrations of different felid species throughout the

oestrous cycle, even within a lineage (Table 2.5) or species (Andrews *et al.*, 2020). Overall, FEM concentrations are approximately four times higher during oestrus than at baseline (interoestrus or anoestrus; Table 2.5; Andrews *et al.*, 2020). It is interesting to note that the basal and peak FEM and FPM concentrations of various species differed considerably between publications (Appendix 2b), perhaps due to the different analytical methods or antibodies used. This large variation resulted in an overlap in the overall (across all publications) means and ranges of the basal and peak FEM and FPM concentrations (Table 2.5; Andrews *et al.*, 2020). Given the level of variability observed, it is unlikely that one-off FEM or FPM analyses could be used to confirm oestrus or dioestrus respectively. Instead, for each analysis method, and potentially each animal, a baseline comparison will be needed to identify oestrus or dioestrus using circulating steroid or faecal steroid metabolite analyses.

The accuracy and reliability of faecal steroid metabolite assays are also affected by several other variables including the amount of time faecal samples are left exposed before collection and bacterial enzymes, both of which can influence the concentrations of immunoreactive steroids (Touma & Palme, 2005). It is likely that these factors, together with the uneven dispersal of faecal steroids within the faeces, could contribute to the highly variable FEM and FPM concentrations observed among felids (Brown *et al.*, 1994; Wielebnowski and Watters, 2007). Care is therefore needed when collecting, processing, and preserving faecal samples in order minimise variation associated with collection.

The practical application of FEM for real-time detection of oestrus is limited. The highly digestible diet of felids means that faecal output is small and often produced intermittently. In addition, group housing makes is difficult to identify which samples came from which animal, although felids are usually separated for feeding so labelling of diets (e.g., use of glitter or food dyes) is possible. The most significant limitation of FEM monitoring for real-time detection of oestrus is time, as there is a 24 h lag between a rise in circulating oestradiol concentrations and the corresponding increase in FEM concentrations (Brown *et al.*, 1994; Graham *et al.*, 1995; Brown and Wildt, 1997). A further delay occurs due to the time taken to prepare and dry the faecal sample, and to subsequently extract and analyse the FEM concentrations (Brown *et al.*, 1994; Brown and Wildt, 1997). The duration of oestrus is short in many felids (less than a few days; Appendix 1b; Andrews *et al.*, 2019), thus oestrus may have ended before any associated rise in circulating oestradiol concentrations is detected via FEM.

2.3.4.2.3 Vaginal cytology

The elevated oestradiol concentrations associated with oestrus are known to cause morphological changes to vaginal epithelial cells (Olğaç *et al.*, 2017). The histological analysis of vaginal smears (i.e., vaginal cytology), has been used for decades to successfully monitor the reproductive cyclicity of domestic cats, with the proportions of exfoliated epithelial cells (e.g., parabasal, intermediate and superficial), and the amount of cellular debris present in the smear differing according to reproductive

stage (Michael and Scott, 1964; Mills *et al.*, 1979; Asa *et al.*, 1992; Zambelli and Cunto, 2005; Chatdarong *et al.*, 2006; Durrant *et al.*, 2006; Malandain *et al.*, 2011; Kanca *et al.*, 2014). In the domestic cat, pro-oestrus smears are characterised by numerous intermediate cells, some nucleated superficial cells, and low levels of non-cellular debris (Mills *et al.*, 1979; Malandain *et al.*, 2011; Kanca *et al.*, 2014). Highly cellular smears with a predominance of nucleated and anucleated superficial epithelial cells (>80%), and absence of non-cellular debris are indicative of oestrus (Mills *et al.*, 1979; Malandain *et al.*, 2011; Kanca *et al.*, 2014). In contrast, smears taken during anoestrus contain comparatively few cells, and those that are present are medium-sized intermediate epithelial cells with a high level of non-cellular debris (Mills *et al.*, 1979; Malandain *et al.*, 2011; Kanca *et al.*, 2014).

Vaginal cytology has been used to monitor the reproductive state of some non-domestic felids (24% of felid species), including the Eurasian lynx (Goeritz *et al.*, 2009; Painer *et al.*, 2014), Iberian lynx (Goeritz *et al.*, 2009), lion (Schmidt *et al.*, 1979), puma (Bonney *et al.*, 1981), cheetah (Asa *et al.*, 1992), and ocelot (*Leopardis pardalis*; Rodrigues da Paz *et al.*, 2005). It is difficult, however, to obtain vaginal smears frequently enough to accurately assess the ovarian activity of these species without compromising the animal's health, wellbeing, and reproductive performance; largely due to the need for restraint or sedation (Schmidt *et al.*, 1979; Asa *et al.*, 1992). Thus, the application of vaginal histology for monitoring ovarian activity in non-domestic felids is limited.

2.3.4.2.4 Laparoscopic examinations

Laparoscopy (the insertion of a fibre-optic camera into the abdomen) has been used to monitor ovarian activity in domestic cats and various non-domestic felids. It offers an accurate and detailed assessment of ovarian structures, follicular growth and development, ovulation, and CL formation, but requires anaesthetisation and minor surgery (i.e., making a puncture incision into the abdominal cavity; Bonney *et al.*, 1981). Thus, the use of laparoscopy for regularly monitoring of ovarian activity is impractical. However, laparoscopy has been used to determine the ovarian response to exogenous gonadotrophins and aid ART in a range of felids including, but not limited to, the domestic cat (Howard *et al.*, 1992b; Roth *et al.*, 1997b; Tsutsui, 2006), Pallas' cat (Swanson, 2006), leopard cat (Tajima *et al.*, 2016), puma (Barone *et al.*, 1994b), cheetah (Howard *et al.*, 1992a; Howard *et al.*, 1997), ocelot (Swanson *et al.*, 1996a), tiger (Donoghue *et al.*, 1996), snow leopard (Roth *et al.*, 1997a), and clouded leopard (Howard *et al.*, 1996; Howard *et al.*, 1997). The use of laparoscopy to guide artificial insemination (AI) procedures has been shown to greatly improve the success of AI (Conforti *et al.*, 2013).

2.3.4.2.5 Transabdominal ultrasonography

Transabdominal ultrasonography is a less invasive method than laparoscopy for assessing the morphology of the ovary, although the sensitivity of the method is highly dependent on the resolution

power of the equipment and technical expertise (Silva *et al.*, 2017). Follicular activity and growth have been assessed accurately in the domestic cat using transabdominal ultrasonography, with ultrasound imagery used for detailed measurements of follicular growth (e.g., information on the number and size of developing ovarian follicles; Malandain *et al.*, 2011; Painer *et al.*, 2014). However, the use of ultrasonography for non-domestic species is limited as it generally requires anaesthesia. Despite this, ultrasonography has been used to monitor the ovarian function of four non-domestic felid species: cheetah (Schulman *et al.*, 2015), Eurasian lynx (Goeritz *et al.*, 2009; Painer *et al.*, 2014), Iberian lynx (Goeritz *et al.*, 2009), and lion (Kirberger *et al.*, 2011; Callealta *et al.*, 2019).

Ultrasound imaging has also been used to evaluate the effectiveness of exogenous hormone treatments for controlling ovarian activity (Painer *et al.*, 2014). Painer *et al.* (2014) used ultrasound examinations to determine if exogenous prostaglandin treatments could cause luteolysis in the seasonally monoestric (i.e., one oestrus event per breeding season) *Lynx* spp., with the aim of inducing a second oestrus event during the breeding season. Ultrasonography has also been used to confirm ovulation following exogenous induction of oestrus and ovulation, and to guide ART such as AI and embryo collection (Silva *et al.*, 2004; Göritz *et al.*, 2012; Callealta *et al.*, 2019).

2.3.4.3 A species-specific account of felid oestrous cycles

The oestrous cycle has been described for only 24 of the 38 felid species, although for many species, information is available on the duration of oestrus only. Furthermore, many studies on the oestrous cyclicity of felids have been conducted on small sample sizes (either in terms of numbers of animals or numbers of events).

The duration of oestrus does not differ significantly among felid lineages (P=0.08) or species (P=0.11), but the addition of more accurate data (e.g., endocrine assessment only) on the oestrous cycles of many felids may yield different results. While there is some intra- and inter-species variation, the duration of oestrus generally ranges from two to 10 days (Table 2.6). Longer periods of oestrus have been observed in some species, such as domestic cats (weighted mean, range, sample size: 7.3 days, range: 1-118, n_E=438), sand cats (*Felis margarita*; 2.9 days, range: 1-11, n_E=109), rusty-spotted cats (5.6 days, range: 1-11, n_E=50), jaguars (6.5 days, <15, n_E=201), snow leopards (4.3 days, range: 1-19, n_E=145) and clouded leopards (5.2 days, range: 1-17, n_E=237), but the mean durations of oestrus for these species still fall within a two to 10-day range.

Despite this, considerable inter- and intra-specific variation in the oestrous cycles of felids is evident, so extrapolation of knowledge across even closely related species is not recommended. Given that the duration of oestrus appears to be relatively consistent, it seems that most of the inter- and intra-specific variation in oestrous cycle length of felids is associated with the highly variable interoestrus intervals. While the mean duration of interoestrus did not differ significantly between lineages (P=0.31) or species (P=0.45), a high degree of variability has been reported for each lineage (Table

2.6) and species. Furthermore, the variability reported for the oestrous cycle length of many felids is likely to be underestimated, as variability appears to increase as more females and cycles are observed, with greater oestrous cycle length variation reported for well-studied species such as the domestic cat and *Panther* spp. In fact, the oestrous cycles of only 15 of the 38 species have been described from sample sizes of five or more animals, even when sample sizes are combined across all relevant publications. This suggests that large sample sizes are critical in describing the reproductive biology of female felids, to ensure that the extreme variability of most feline oestrous cycles is captured.

Table 2.6 Weighted means (in days) of female reproductive parameters of the eight felid lineages. The values presented have been calculated from Appendix 1b. *Excludes the lynx lineage. Abbreviations: non-pregnant luteal phase (NPLP), no data (ND).

Lineage	Mean length of anovulatory oestrous cycle, in days (range)	Mean duration of oestrus, in days (range)	Range of inter-oestrus interval, in days	Mean duration of NPLP, in days (range)	Mean duration of gestation, in days (range)	NPLP as % duration of gestation
Domestic cat	14.5 (2 – 69)	5.8 (1 – 118)	2 – 65	32.2 (12 – 55)	65.7 (60 – 71)	48.7
Leopard cat	20.4 (7 – 43)	6.0 (1 – 11)	1 – 39	34.8 (18 – 60)	68.9 (56 – 76)	50.2
Puma	14.2 (3 – 55)	4.1 (1 – 9)	3 – 50	51.7 (38 – 62)	92.8 (72 – 98)	55.9
Lynx	Monoestric	5.0 (1 – 10)	Monoestric	>2.0 years	65.7 (60 – 72)	>1111.0
Ocelot	18.7 (1 – 52)	3.2 (1 – 6)	6 – 60	35.6 (28 – 60)	72.2 (66 – 83)	44.6
Caracal	18.3 (4 – 54)	4.5 (3 – 6)	13 – 51	47.5 (47 – 48)	78.2 (75 – 81)	60.3
Bay cat	39.0	6.0	~33	ND	79.0 (74 – 84)	ND
Panthera	21.4 (5 – 148)	5.0 (1 – 19)	1 – 145	43.8 (11 – 72)	101.8 (50 – 127)	44.1
Overall mean	17.7 (1 – 148)	5.2 (1 – 118)	1 – 145	40.9 (11 – 62)*	78.0 (50 – 127)	47.6*

Spontaneous ovulations have been observed in all felid lineages (except the caracal and bay cat lineages, for which there is a general paucity of published research on reproductive biology). The frequency of spontaneous ovulations appears to differ considerably between felid species. Non-pregnant luteal phases have been described for 19 of the 38 felid species. The duration of NPLP has typically been reported to be about one-third that of gestation (Brown, 2011); however, our more comprehensive literature analysis indicates that the duration of NPLP typically persists for about half the duration of PLP (48%, range: 22-71%, n_E=256; Appendix 1c). The exception is the *Lynx* spp. which exhibit prolonged NPLP, with CL and elevated progesterone concentrations persisting for two years or more (Goeritz *et al.*, 2009; Jewgenow *et al.*, 2014; Painer *et al.*, 2014). Female *Lynx* spp. can re-enter oestrus during subsequent breeding seasons since old CL are structurally present, but functionally suppressed, until after the breeding season (Jewgenow *et al.*, 2014; Painer *et al.*, 2014). Pregnant luteal phases are also prolonged (for two years or more) in this lineage, despite a gestation length of 60 to 70 days (Jewgenow *et al.*, 2014; Painer *et al.*, 2014).

The duration of gestation differs considerably within Felidae (P<0.001), but is generally similar within each lineage (P=0.2; Table 2.6). Nowell and Jackson (1996) categorised felids according to body type into small (<6.5 kg), medium (7-20 kg), and large (35-135 kg) species. Weighted means of

the gestation periods of these groups support the hypothesis that larger cats have longer gestation periods ($P<0.001$), with small, medium, and large cats exhibiting different ($P<0.05$) mean gestation lengths of 67.8 days (range: 60-84, n_E = 79), 79.4 days (range: 60-121, n_E = 138), and 98.3 days (range: 50-127, n_E = 504), respectively.

Factors that appear to drive variation in felid oestrous cycle length include the captive environment (enclosure size or level of enrichment), time spent with keepers, number of veterinary treatments, body weight, age, diet, and season (Setchell *et al.*, 1987; Mellen, 1991; Swanson *et al.*, 1996b; Brown *et al.*, 2002; Rodrigues da Paz *et al.*, 2006; Fanson *et al.*, 2010; Balme *et al.*, 2013). Many felid species are highly seasonal with respect to the frequency of oestrus (i.e., the length of interoestrus; Foreman, 1997; Moreira *et al.*, 2001; Brown *et al.*, 2002; Kinoshita *et al.*, 2009). Most studies have been conducted on captive animals, but the captive environment has been shown to affect reproductive seasonality (Swanson *et al.*, 1996b; Foreman, 1997; Brown *et al.*, 2002). For example, Geoffroy's cats are thought to be seasonal, monoestrous breeders in the wild, but have been found to cycle all year round in captivity (although the frequency of oestrus still peaks between February and August in the northern hemisphere; Foreman, 1997). The increased ovarian cyclicity of Geoffroy's cats in captivity is thought to be associated with a more consistent food supply (Foreman, 1997). Many wild felids live at high altitudes or latitudes, where prey densities fluctuate seasonally (Johnston *et al.*, 1994; Swanson *et al.*, 1996b; Brown *et al.*, 2002; Newell-Fugate *et al.*, 2007; Göritz *et al.*, 2009), but this is seldom mirrored in feeding regimes for captive-held felids. However, if food availability or body weight were the only factors regulating seasonal reproduction in felids, then all felid species would be expected to breed all year round in captivity (as their diet is consistent), but this is not the case (Schmidt *et al.*, 1993; Swanson *et al.*, 1996b; Brown *et al.*, 2002; Göritz *et al.*, 2009).

There is considerable evidence for photoperiod-regulated reproduction in felids (Shille *et al.*, 1979; Michel, 1993; Brown *et al.*, 2002; Graham *et al.*, 2004; Gimenez *et al.*, 2009). Artificial lighting has been shown to stimulate follicular development in Pallas' cats temporarily, during the non-breeding season (Swanson *et al.*, 1996b; Brown *et al.*, 2002). Both artificial lighting and exogenous melatonin treatment can influence the frequency of oestrus in domestic cats (Michel, 1993; Graham *et al.*, 2004; Tsutsui *et al.*, 2004; Gimenez *et al.*, 2009). While this seemingly provides putative support for the photoperiodic control of seasonal reproduction in felids, factors such as body weight, temperature, prey availability, and social stimuli have also been associated with seasonal reproduction and cannot be ignored (Michel, 1993; Swanson *et al.*, 1996b; Foreman, 1997; Brown *et al.*, 2002; Jansen and Jenks, 2012). Regardless of these other factors, it is clear that seasonality may contribute towards the highly variable oestrous cycles described in many felids by altering the frequency of oestrus (i.e., duration of interoestrus).

2.3.3 Factors affecting the reproductive performance of felids

2.3.3.1 A lack of knowledge about the reproductive biology

In general, reproduction-focussed literature is limited for smaller felids, with data showing a strong positive correlation between body size and research effort (Brodie 2009, Brooke *et al.*, 2014). This may be because larger cats are more commonly held in captivity, and thus are more accessible for researchers (Brodie 2009, Inskip & Zimmermann 2009, Brooke *et al.*, 2014). Indeed, the species for which reproductive biology literature are limited are rarely held in captivity, are restricted to low-density populations in the wild, and are generally cryptic and difficult to study (Nowell and Jackson, 1996; Sunquist and Sunquist, 2002; Brodie, 2009; Brooke *et al.*, 2014; IUCN, 2020). However, peer-reviewed literature on caracals and servals is also lacking, despite an abundance of these species in captivity, so it may be more accurate to state that research is focussed on the more popular and iconic species (e.g., *Panthera* spp. and cheetah; Figure 2.2).

It is evident that further research into the reproductive biology of many felid species is required to optimise the success of both captive breeding programs and *in situ* conservation strategies. Furthermore, studies investigating the reproductive biology of felids should be conducted on larger sample sizes to capture the high degree of variability in the oestrous cycles and ejaculate characteristics of felids.

2.3.3.2 Loss of genetic diversity

The progressive loss of GD has been identified as one of the major challenges for felid conservation (Obrien *et al.*, 1985; Facemire *et al.*, 1995; Nowell and Jackson, 1996). The effects of low GD on fertility have been studied more in male felids than in females (Wildt *et al.*, 1987a; Barone *et al.*, 1994a). Cheetahs, for example, experienced a major genetic bottleneck approximately 10,000 years ago and are now almost genetically monomorphic (Obrien *et al.*, 1985; Menotti-Raymond and O'Brien, 1993; Pukazhenthi *et al.*, 2006b). The high prevalence of teratospermia in this species may be linked to low GD, but a causal link is difficult to prove due to a lack of more genetically diverse populations for comparison. However, data from other species support a causal relationship (Brown *et al.*, 1991; Pukazhenthi *et al.*, 2006b). For instance, lions from populations with poor GD produce ejaculates with a higher proportion of pleiomorphic sperm than lions from populations with better GD (Wildt *et al.*, 1987a; Brown *et al.*, 1991). Interestingly, a single generation of inbreeding is sufficient to decrease semen quality in dogs (*Canis familiaris*; Wildt *et al.*, 1982). If this is similar for felids, it is particularly concerning as it suggests that the negative effects of inbreeding can occur rapidly and is especially problematic given the severity of felid habitat loss and fragmentation (Nowell and Jackson, 1996; Wolf and Ripple, 2017). It is unlikely, however, that poor GD is the only cause of the high prevalence of teratospermia within Felidae, since some species (e.g., Canadian lynx (*Lynx Canadensis*), Eurasian lynx, and domestic cats) produce teratospermic ejaculates despite reasonably high GD (Schwartz *et al.*, 2003; Swanson, 2003; Pukazhenthi *et al.*, 2006b; Schmidt *et al.*, 2011).

2.3.3.3 Challenges with the detection of oestrus

As outline earlier, the accurate and reliable detection of oestrus has been difficult to achieve in a number of species due to a lack of overt behavioural indicators of oestrus (Foreman, 1997; Wielebnowski and Brown, 1998; Moreira *et al.*, 2001; Brown *et al.*, 2002; Henriksen *et al.*, 2005). There is currently no single non-invasive 'best method' for detecting oestrus in felids (Table 2.7), and it is likely that best practice would involve a combination of existing methods. The combined use of transabdominal ultrasonography, serum progesterone monitoring, and vaginal cytology has been proposed as a "reasonably accurate" means of monitoring ovarian cyclicity of captive cheetah (Schulman *et al.*, 2015) and lions (Kirberger *et al.*, 2011), but this requires sedation/anaesthetisation. Consequently, non-invasive techniques such as FEM and FPM monitoring remain the most convenient sampling options, although the time-lag between sampling and results limits their relevance for management decisions. Researchers still urgently require a non-invasive (i.e., low stress) method that can be used for the real-time detection of oestrus (Table 2.7).

Table 2.7 Evaluation of the methods used to monitor follicular growth and/or detect oestrus in felids. For each method, the parameters are marked as either '✓' meets parameter, 'X' does not meet parameter, 'ND' no data for felids. An ideal method would meet all the hypothetical parameters. Abbreviations: faecal oestradiol metabolites (FEM), faecal progesterone metabolites (FPM), infrared (IR), oestradiol (E_2).

Method	Parameters of an ideal method				Notes
	Works for all or most felid species	Accurate and reliable	Quick assessment	Non-invasive	
Ovarian function					
Behaviour					
Observed	X	X	✓	✓	Silent oestrus common.
Accelerometry	ND	ND	✓	✓	Effective in non-felid species
Endocrinology					
Blood E_2	✓	✓	✓	X	-
FEM/FPM	✓	✓	X	✓	Retrospective monitoring
IR thermography	ND	ND	✓	✓	Effective in non-felid species
Laparoscopy	✓	✓	✓	X	-
Ultrasonography	✓	✓	✓	X	-
Vaginal cytology	✓	✓	✓	X	-

While the visual detection of oestrus is challenging in many felid species due to a lack of overt behaviour indicators of oestrus, it does not necessarily mean that oestrus is silent (with no behavioural changes) in these species (Asa *et al.*, 1992; Graham *et al.*, 1995; Foreman, 1997; Wielebnowski and Brown, 1998; Moreira *et al.*, 2001; Brown *et al.*, 2002). Instead, oestrus is likely to be associated with subtle increases in behaviours such as locomotion, rubbing, rolling, sniffing, vocalisation, grooming and scent-marking (Asa *et al.*, 1992; Graham *et al.*, 1995; Foreman, 1997; Wielebnowski and Brown, 1998; Moreira *et al.*, 2001; Brown *et al.*, 2002). These subtle changes are unlikely to be detected without detailed and labour-intensive behavioural assessment, which is further complicated by the cryptic and crepuscular/nocturnal nature of most felids. However, this may mean that there is an opportunity to develop technologies that can automatically and continuously monitor the behaviour of

felids (e.g., accelerometry), and thus be used to monitoring the reproductive state of felids (see section 2.7).

2.3.3.4 Highly variable oestrous cycles

The high degree of variability of natural felid oestrous cyclicity further complicates monitoring the ovarian cycles of felids and provides support for the use of exogenous gonadotrophins to stimulate follicular growth (e.g., equine chorionic gonadotrophin) and trigger ovulation (e.g., human chorionic gonadotrophin), especially for the purpose of ART (Thongphakdee *et al.*, 2018; Thongphakdee *et al.*, 2020). However, the responses of felids to exogenous gonadotrophin treatment also appear to be highly variable (Thongphakdee *et al.*, 2018). Thongphakdee *et al.* (2018) stated that the highly variable ovarian response of felids to exogenous gonadotrophins is a "major restriction" for ART. Pre-treatment with a follicular inhibitor (e.g., levonorgestrel or altrenogest) has been shown to result in a more consistent ovarian response (i.e., greater follicular development) to exogenous gonadotrophins (Pelican *et al.*, 2003; Pelican *et al.*, 2006; Pelican *et al.*, 2008; Pelican *et al.*, 2010; Stewart *et al.*, 2012). A more consistent ovarian response could also be achieved by stimulating follicular growth during the non-breeding season, when all follicles are undeveloped, although this remains to be tested in felids.

The effect of seasonality on reproductive activity should also be considered when breeding felids in captivity, since season can affect ovarian cyclicity and responsiveness to exogenous gonadotrophins (Thongphakdee *et al.*, 2018). It is important in the context of captive management and breeding that felids are exposed to a photoperiod that they would experience in their natural geographic range, since moving felids outside their natural latitudinal range (i.e., seasonal photoperiod) may affect their seasonality and thus reproductive performance.

2.3.3.5 Method used for sperm collection

The method used to collect ejaculates/sperm samples is also important. Electroejaculation is by far the most commonly used method to collect sperm from felids, but UC with pharmacological induction (i.e., intramuscular injection of medetomidine) yields much higher sperm concentrations (Figure 2.3). The natural compensatory mechanism for teratospermia appears to be the production of an ejaculate with higher concentrations (and a higher total number) of sperm (Müller *et al.*, 2012), thus UC may be a better method for collecting sperm from felids. To date, UC has been used to collect sperm from only four felid species. Further investigation into the use of this method as an alternative to EE is required. Irrespective of collection method or seasonality, teratospermia is likely to remain a significant problem for many felid species. Since low GD is likely to be a major cause of teratospermia in felids, it is important that efforts to reduce further losses of GD are prioritised, especially for species facing severe habitat fragmentation and thus the formation of genetically isolated subpopulations. A long-term management goal for both *in situ* and *ex situ* conservation efforts for felids must be to maintain and enhance GD.

2.3.3.6 Captivity-related stress

Felids appear to be particularly sensitive to captivity-related stress (Swanson, 2003). Captive clouded leopards, tigers, cheetah, fishing cats, and lions all display higher frequencies of stress-related behaviours (e.g., stereotypies, excessive sleep, aggression, hiding, reduced exploration, reduced appetitive behaviour and self-mutilation) than their wild counterparts (Wielebnowski *et al.*, 2002a; Bashaw *et al.*, 2003; Terio *et al.*, 2004; Szokalski *et al.*, 2012). A more quantitative means of assessing stress is to analyse basal circulating glucocorticoids (GC) concentrations, as GC are synthesised and secreted in response to stress. Interestingly, captive felids have been found to exhibit higher basal glucocorticoid (GC) concentrations than their wild conspecifics, with faecal GC metabolite (FGM) concentrations being ~2.5 times higher in captive cheetah, Canadian lynx, and clouded leopards than in their wild conspecifics (Wielebnowski *et al.*, 2002a; Terio *et al.*, 2004; Fanson *et al.*, 2010).

It is likely that the elevated GC concentrations observed in captive felids adversely affects their reproductive performance. Many felids have certainly been found to reproduce less successfully in captivity (Swanson, 2003; Terio *et al.*, 2004; Fanson *et al.*, 2010). For example, the captive cheetah population is not self-sustaining, with only a small proportion (~20%) of the captive population having ever bred successfully (Marker, 2012; Grisham *et al.*, 2013). Jurke *et al.* (1997) found that non-cycling female cheetah display significantly higher FGM concentrations than cycling cheetah; moreover, it was found that non-cycling cheetah would commence cycling if they experienced a period of lowered GC concentrations following enclosure enrichment. Captive margays (*Leopardus weidii*) and tigrinas (*Leopardis tigrinus*) housed in small, barren (i.e., no enrichment) enclosures exhibit elevated FGM concentrations and poor ovarian cyclicity; interestingly, the movement of these margay and tigrinas to larger, more enriched enclosures lead to a decrease in FGM concentrations and the recommencement of normal ovarian cyclicity (Moreira *et al.*, 2007). Similarly, captive female bobcats failed to breed in enclosures lacking enrichment (Mollá *et al.*, 2011).

Captivity has also been linked to reduced testicular function in felids, with captive cheetah exhibiting markedly (~ four times) lower testosterone concentrations than their wild conspecifics (Table 2.4; Andrews *et al.*, 2019). Reduced testosterone concentrations are likely to adversely affect the ejaculate quality of felids. However, there may also be other pathways involved, as cheetahs on public display or with more than three carers produce ejaculates that have lower sperm motility than those off public display with fewer carers, but had comparable testosterone concentrations (Koester *et al.*, 2015).

It seems evident that captivity adversely affects both the ovarian and testicular function of felids, and also leads to an increase in basal circulating GC concentrations. Glucocorticoids are thought to be the main link between the HPA and HPG axes, although several other neurological links exist (Tellam *et al.*, 2000; Gore *et al.*, 2006). It has been shown that GC act on all levels of the HPG axis to suppress

ovarian and testicular function in mammals (Hsueh and Erickson, 1978; Bambino and Hsueh, 1981; Welsh *et al.*, 1982; Orr and Mann, 1992; Sharpe *et al.*, 1992; Tetsuka *et al.*, 1999; Yang *et al.*, 1999; Gore *et al.*, 2006; Van Merris *et al.*, 2007; Kirby *et al.*, 2009). However, there has been no direct research to determine if the elevated GC associated with captivity-related stress are the cause for the reduced reproductive performance of felids in captivity.

2.4 Stress and reproduction

2.4.1 Defining stress and stressors

For the purpose of this thesis, stress is defined as the activation of the HPA axis in response to any predicted threat or physical challenge to homeostasis (Miller and O'Callaghan, 2002). In a physiological context, stressful stimuli, or stressors, can be considered as either (1) reactive/physical or (2) anticipatory/emotional (Table 2.8; Herman *et al.*, 2003). Reactive stressors stimulate the HPA axis in response to a homeostatic challenge, while anticipatory or emotional stressors have no immediate reactive effect on the animals, but activate the HPA axis in the anticipation of a homeostatic challenge (Herman *et al.*, 2003). The anticipatory stress response is dependent on the learned (e.g., past experiences, memory, social learning, conditioning etc.) or innate awareness of the potentially disruptive effects of a given stimulus on homeostasis (Table 2.8; Herman *et al.*, 2003). Most stressors associated with captivity-related stress are anticipatory stress and can be categorised as either abiotic environmental stressors (AES) or confinement-specific stressors (CSS; Morgan and Tromborg, 2007).

Table 2.8 Some examples of reactive and anticipatory stressors and the signals pathways via which they stressors stimulate the HPA axis.

Type of stressor		Stressors	Examples of signals and stimuli perception
Reactive or physical stressors		Pain (somatic or visceral)	Somatic or visceral nociceptors
		Cardiovascular tone	Baroreceptors
		Respiratory distress	Chemoreceptors
		Obesity/starvation	Plasma glucose, leptin and/or insulin concentrations
		Water balance	Osmoreceptors
			Hormones (ADH, ANP, renin-angiotensin)
		Disease and illness	Immune signals (cytokine like interleukins and chemokine factors)
		Hypo- or hyperthermia	Thermoreceptors
Anticipatory stressors	**Innate**	Presence of a predator	Somatic sensory stimuli
		Novel situations or objects	Somatic sensory stimuli
		Social challenges	Somatic sensory stimuli
	Memory	Past experiences	Somatic sensory stimuli
		Conditioned stimuli	Somatic Sensory stimuli
		Negative reinforcement training	Somatic Sensory stimuli

2.4.2 The hypothalamic-pituitary-adrenal or stress axis

Both AES and CSS culminate in the activation of the HPA axis, which is widely regarded as the central stress response system. The complex neurological pathways by which stress up-regulates the activity of corticotrophin-releasing hormone (CRH) neurons in the paraventricular nucleus (PVN) of the hypothalamus, and thus the HPA axis, are considered in Appendix 3 (Aguilera, 1998; Miller and O'Callaghan, 2002). Approximately 50 to 90% of CRH neurons in the PVN project into the medial eminence (ME) and secrete CRH into the hypophyseal-portal blood, with CRH subsequently acting on corticotroph cells in the anterior pituitary via corticotrophin-release hormone receptor 1 (CRH-R1; Owens and Nemeroff, 1991; Aguilera, 1998; Miller and O'Callaghan, 2002). The binding of CRH to CRH-R1 activates an AC and PKA signalling pathway that ultimately upregulates the synthesis of proopiomelanocortin (POMC), which is a pro-hormone that is cleaved to form a number of hormones including adrenocorticotrophic-releasing hormone (ACTH; Bonfiglio *et al.*, 2011).

Adrenocorticotrophic-releasing hormone is the main product of corticotroph cells and acts via the melanocortin 2 receptor to stimulate the synthesis and secretion of GC from cells in the zona fasciculata of the cortex of the adrenal gland (Miller and O'Callaghan, 2002; Gallo-Payet and Payet, 2003). The binding of ACTH to melanocortin 2 receptor and activates the G_s-protein that triggers AC to produce cAMP and, in turn, stimulate a PKA signalling pathway (Gallo-Payet and Payet, 2003). Protein kinase A acts directly and indirectly (via a rise in intracellular Ca^{2+}) to promote the synthesis and secretion of GC (Gallo-Payet & Payet, 2003).

Glucocorticoids (e.g., cortisol and corticosterone) are the 'end products' of the HPA axis. As with other steroid hormones, GC are membrane permeable and act via intracellular receptors (Lowe *et al.*, 2008b). Inactive glucocorticoid receptors (GR) are bound to chaperone proteins that prevent the receptor from entering the nucleus (Lowe *et al.*, 2008b). The binding of GC to GR leads to a conformational change that releases the chaperone proteins and enables the GR-GC complex to cross the nuclear membrane (Lowe *et al.*, 2008b). Once in the nucleus, GR-GC complexes form homodimers (i.e., two GR-GC complexes combined) and bind to glucocorticoid response element regions of DNA to promote or inhibit transcription (Lowe *et al.*, 2008b). The pathways by which GC exert non-genomic effects on cells are less clear, but are likely mediated by the direct effects of GC in the cytosol, cytosolic GC-GR complexes, GR chaperone proteins, and/or membrane-bound GRs (Lowe *et al.*, 2008b).

2.4.3 The effects of stress and the activated HPA axis on the HPG axis

The main pathways by which the HPA axis acts to suppress the HPG axis are summarised in Figure 2.6. For a comprehensive diagram illustrating all of the pathways, as well as the intracellular mechanism by which the HPA axis supresses the HPG axis, see Appendix 4.

2.4.3.1 At the level of the hypothalamus

Gonadotrophin-releasing hormone neurons in the POA of the rat (*Rattus novegicus*) hypothalamus express functional type two GR, and corticosterone implants have been found to greatly decrease GnRH mRNA expression in the rat (Gore *et al.*, 2006). However, GC administration does not appear to decrease GnRH concentrations in the hypophyseal-portal blood of rats and ewes (Breen and Karsch, 2006; Kirby *et al.*, 2009). Perhaps the response of GnRH neurons to GC is species-specific, although contradictory results have been reported within a single species, the rat (Gore *et al.*, 2006; Kirby *et al.*, 2009).

It is possible that the effects of stress on hypothalamic GnRH neurons are not mediated by GC. The POA and suprachiasmatic nucleus regions, rich in GnRH neuronal perikarya, express both CRH-R1 and corticotrophin-release hormone receptor 2_{α} (Cummings *et al.*, 1983; Merchenthaler *et al.*, 1984; Palchaudhuri *et al.*, 1998; Palchaudhuri *et al.*, 1999; Chen *et al.*, 2000). Thus, the effects of stress on hypothalamic GnRH neurons may be mediated via CRH neurons rather than GC (Figure 2.6). Indeed, CRH-containing nerve terminals synapse directly with GnRH neurons within the POA of the rat hypothalamus (Maclusky *et al.*, 1988). Both CRH and CRH-R are also highly expressed within the ME, thus CRH may also acts on the GnRH nerve terminals located there (Palchaudhuri *et al.*, 1998; Palchaudhuri *et al.*, 1999; Chen *et al.*, 2000). An infusion of CRH into the third ventricle of the brain leads to a substantial decrease in GnRH expression within the ME of reproductively active (in follicular phase) ewes (Ciechanowska *et al.*, 2011). An infusion of CRH directly into the POA of rats also results in a significant decrease in the amount of GnRH released into the ME (Rivier and Rivest, 1991). Corticotrophin-releasing hormone also inhibits the release of GnRH from rat hypothalamus cells *in vitro*, although it is unclear whether this is due to the direct effects of CRH on GnRH neurons or if intermediate neurons are involved (Gambacciani *et al.*, 1986; Nikolarakis *et al.*, 1986).

The effects of both CRH and GC on hypothalamic GnRH neurons may also be mediated through intermediate neurons such as kiSS and/or GnIH neurons (Figure 2.6). Kisspeptin is a potent stimulator of hypothalamic GnRH neurons, acting via the G-protein-coupled receptor-54 (GPR-54) to stimulate both the synthesis and secretion of GnRH (Colledge, 2009). The perikarya of kiSS neurons are primarily located in the ARC and anteroventral periventricular nucleus (AVPV) of the hypothalamus (Greives *et al.*, 2007; Takumi *et al.*, 2012). Corticotrophin-releasing hormone neurons and fibres have been identified in the ARC, and to a lesser extent, the AVPV (Cummings *et al.*, 1983). Furthermore, 99% of the kiSS neurons in the ARC of the rat hypothalamus express CRH-Rs and are inhibited by CRH (Takumi *et al.*, 2012). Kisspeptin is required to maintain a functional HPG axis, since mice (*Mus musculus*) lacking kiSS or GPR-54 (gene knockout mice) exhibit hypotrophic hypogonadism (Lapatto *et al.*, 2007; Roseweir and Millar, 2009). As such, the suppression of kiSS neurons by CRH would be expected to greatly affect the function of the HPG axis.

Gonadotrophin-inhibitory hormone neurons in the dorsomedial nucleus of the hypothalamus also appear to be particularly important in mediating the effects of GC on GnRH neurons (Figure 2.6; Ducret *et al.*, 2009; Kirby *et al.*, 2009). Approximately 50% and 10% of the GnIH neurons express GR and CRH-R1 respectively; moreover, the majority of these GnIH neurons project into the POA and ME to strongly inhibit GnRH neurons (Ducret *et al.*, 2009; Kirby *et al.*, 2009). Interestingly, chronic stress has been found to greatly increase GnIH mRNA expression within the rat hypothalamus, and even increase the number of GnIH neurons (Kirby *et al.*, 2009). A similar response is observed in rats exposed to acute stress, although the effects are more short-lived (Kirby *et al.*, 2009). However, there is evidence to suggest that the effects of GnIH upregulation on the HPG axis are more likely mediated at the level of the anterior pituitary (Clarke *et al.*, 2008; Smith and Clarke, 2010).

2.4.3.2 At the level of the pituitary

Hypothalamic GnIH neurons, which are up-regulated by GC and CRH, release GnIH into the hypophyseal-portal system (Smith and Clarke, 2010). Thus, it is likey that GnIH also acts directly on pituitary gonadotroph cells to suppress the HPG axis. Gonadotroph cells certainly express the G-protein-coupled receptor 147 (GPR-147), which is the only receptor for GnIH (Bentley *et al.*, 2009). Clarke *et al.* (2008) demonstrated that GnIH dose-dependently inhibits the GnRH-induced production of LH, and to a lesser extent FSH, by ovine gonadotroph cells *in vitro*. However, the effect of GnIH on gonadotroph cells is more difficult to demonstrate *in vivo*, since GnIH also acts on GnRH neurons to suppress the HPG axis. A similar challenge arises when investigating the direct effects of GC on pituitary cells.

Breen and Karsch (2004; 2006) suggested that cortisol/GC act primarily at the level of the anterior pituitary to inhibit the secretion of gonadotrophins. Gonadotroph cells express type two GR and are suppressed by GC *in vitro*, although GC only appear to directly affect the synthesis and secretion of LH (Kononen *et al.*, 1993; Breen and Karsch, 2004; Gore *et al.*, 2006; Whirledge and Cidlowski, 2010). Nonetheless, exogenous GC have been found to decrease plasma LH and FSH concentrations if administered to female rats during the pre-ovulatory GnRH/LH surge (Gore *et al.*, 2006). It is possible that FSH concentrations are affected by pathways other than the direct effects of GC on gonadotrophin cells (e.g., decreased GnRH or kiSS activity and/or increased GnIH activity), although more research is needed to confirm this.

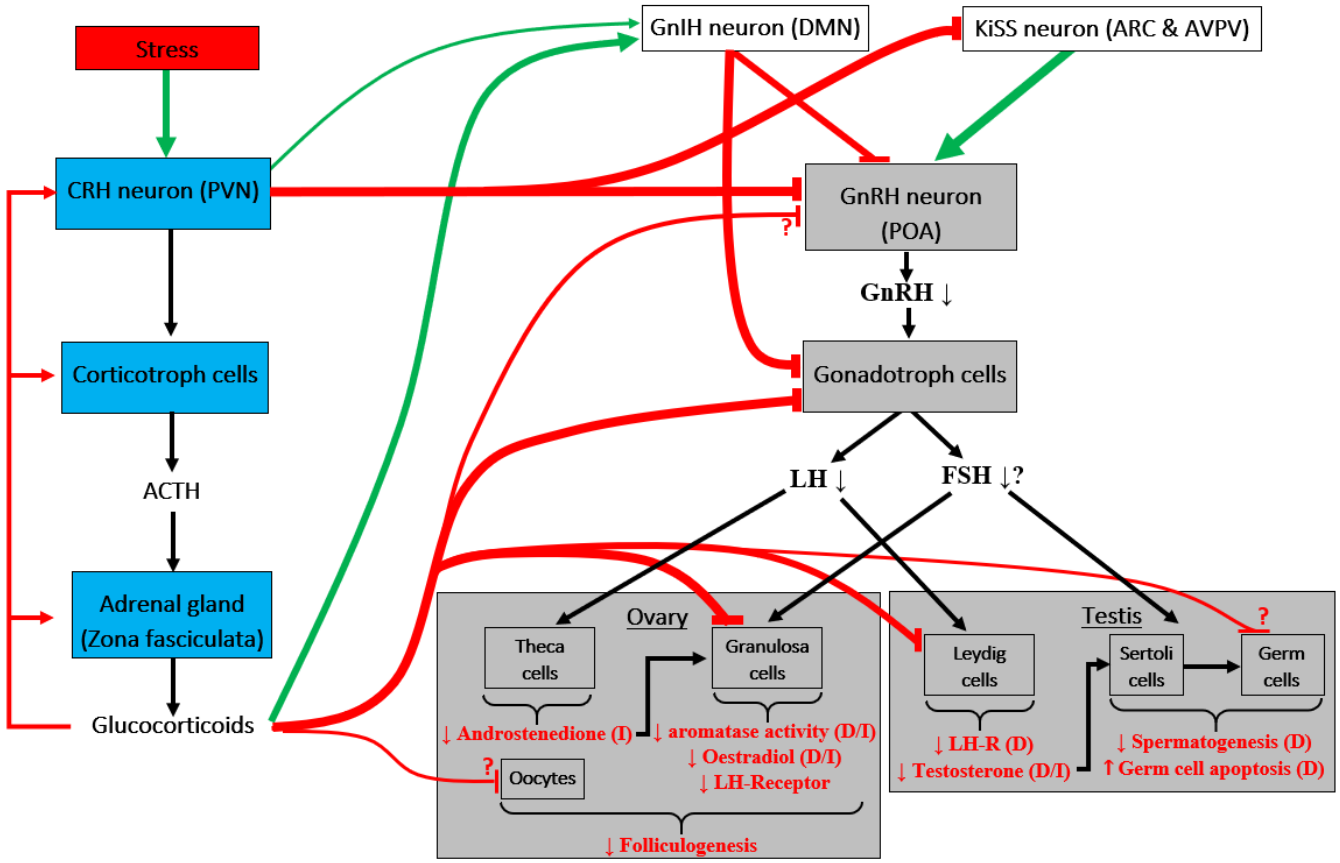

Figure 2.6 The main pathways by which the hypothalamic-pituitary-adrenal (HPA; blue) axis suppresses the hypothalamic-pituitary-gonadal (HPG; grey) axis in mammals. Green lines with arrow ends indicate a stimulatory pathway, red lines with bar ends indicate an inhibitory pathway, and red lines with arrow ends indicate a negative feedback loop. The thickness of the lines provides a relative indication of the importance of each pathway. A question mark indicates a proposed (uncertain) pathway or effect of elevated HPA function on the HPG axis. For a detailed diagram of these pathways and the associated intracellular signalling pathways, please see Appendix 4. Abbreviations: adrenocorticotropic hormone (ACTH) anteroventral periventricular nucleus of the hypothalamus (AVPV), arcuate nucleus of the hypothalamus (ARC), corticotrophin releasing hormone (CRH), direct effect (D), dorsomedial nucleus of the hypothalamus (DMN), follicle stimulating hormone (FSH), gonadotrophin-inhibitor hormone (GnIH), gonadotrophin releasing hormone (GnRH), indirect effect (I), kisspeptin (kiSS), luteinising hormone (LH), luteinising hormone receptor (LH-R), preoptic area of the hypothalamus (POA), paraventricular nucleus of the hypothalamus (PVN).

2.4.3.3 At the level of the gonads

Gonadal functions are predominantly under the control of LH and FSH, thus stress-induced changes in plasma gonadotrophin concentrations will affect gonadal activity (Whirledge and Cidlowski, 2010). In fact, most of the inhibitory effects of stress on ovarian and testicular function appear to be mediated at the level of the hypothalamus and pituitary; although, both the ovaries and testes of rats have been shown to express GR, so the direct effects of GC on the gonads must also be considered (Schreiber *et al.*, 1982; Schultz *et al.*, 1993).

2.4.3.3.1 The testis

In the male rat, the GR appears to be highly expressed by Leydig cells, but not Sertoli cells (Levy *et al.*, 1989; Schultz *et al.*, 1993). The location of GR within the testis suggests that GC act to suppress testosterone production, as that is the main role of Leydig cells. Both dexamethasone and corticosterone dose-dependently inhibit the LH-stimulated secretion of testosterone by the testicular interstitial cells of rats *in vitro* (Bambino and Hsueh, 1981; Welsh *et al.*, 1982; Orr and Mann, 1992). Welsh *et al.* (1982) found that GC suppress Leydig cell functions by inhibiting AC. Given that LH predominantly acts via a AC-cAMP signalling pathway, it is not surprising to find that GC inhibit the LH-induced synthesis of the steroidogenic enzymes (e.g., 17α-hyroxylase, 17-20-lyase, and 17β-hydroxy steroid dehydrogenase) responsible for testosterone production, as GC inhibit this pathway (Welsh *et al.*, 1982; Whirledge and Cidlowski, 2010). Glucocorticoids seem to further decrease the responsiveness of Leydig cells to LH by down-regulating the expression of the LH-receptor by Leydig cells (Bambino and Hsueh, 1981).

While GC have been shown to decrease circulating LH concentrations in male rats, FSH concentrations appear to be unaffected (Gore *et al.*, 2006). As such, any effects of GC administration on spermatogenesis are likely to be partly-mediated by the direct effects of GC on the testis. The decreased testosterone concentrations associated with high GC concentrations would adversely affect certain stages of spermatogenesis, since testosterone has an important regulation role in late spermatogenesis (Welsh *et al.*, 1982; Orr and Mann, 1992; Sharpe *et al.*, 1992). Yazawa *et al.* (2000) found that rats treated with dexamethasone exhibit a noteworthy increase in the apoptosis of primary spermatocytes and spermatids. This is possibly due to the decreased testosterone concentrations associated with the administration of exogenous GC. The treatment of rats with ethane di-methane sulphonate, a compound that destroys Leydig cells and, as a result, testosterone production, leads to increased germ cell degeneration during late spermatogenesis (Sharpe *et al.*, 1992). Furthermore, the effects of ethane di-methane sulphonate can be prevented through testosterone supplementation (Sharpe *et al.*, 1992).

Long-term suppression of testosterone is required to disrupt spermatogenesis, with the effects of GC on Leydig cells being quickly reversible (Welsh *et al.*, 1982; Sharpe *et al.*, 1992; Yazawa *et al.*,

2000). In this context, only chronic stress would be likely to affect spermatogenesis. Even in the case of chronic stress, only a relatively small proportion of germ cells are significantly impacted by the low serum testosterone concentrations (Welsh *et al.*, 1982; Sharpe *et al.*, 1992; Yazawa *et al.*, 2000). Thus, the physiological significance of GC-induced germ cell apoptosis, either directly on spermatocytes (which also express GR) or via decrease testosterone concentrations, is unclear.

Abnormal testosterone concentrations can lead to an increase in primary sperm abnormalities, as testosterone is required for germ cell-Sertoli cell adhesion (Mruk and Cheng, 2004; Wang *et al.*, 2006). Androgen receptor (AR)-knockout mice only exhibit one of the eight proteins involved in the desmosome junction between germ cells and Sertoli cells; these incomplete desmosome junctions have been linked to impaired Sertoli cell function, incomplete sperm development and, in turn, a high proportion of morphologically abnormal sperm (Mruk and Cheng, 2004; Wang *et al.*, 2006). Primary sperm abnormalities are often due to impaired Sertoli cell function and/or high Sertoli cell loads (i.e., too many germ cells per Sertoli cell; Mruk and Cheng, 2004; Pukazhenthi *et al.*, 2006b). Testosterone is also required for the process of spermiation, the release of spermatozoa into the lumen of the seminiferous tubule from Sertoli cells, with an absence of testosterone preventing the process occurring and the unreleased spermatozoa being phagocytised by the Sertoli cells to which they are bound (Walker, 2010). Secondary sperm abnormalities, which occur after spermiation, are also more prevalent when androgen concentrations are abnormal (Meistrich *et al.*, 1975). In rats, a lack of testosterone greatly increases the speed at which spermatozoa move through the epididymis (as testosterone slows sperm transport to enable maturation), which leads to an increase in the proportion of morphologically abnormal sperm (Meistrich *et al.*, 1975). Thus, GC-induced hypoandrogenism can adversely affect sperm production and maturation.

2.4.3.3.2 The ovary

The ovarian granulosa cells of rats have been found to express GR throughout the oestrous cycle; moreover, these GR have a high affinity for GC (Hsueh and Erickson, 1978; Schreiber *et al.*, 1982; Tetsuka *et al.*, 1999). Van Merris *et al.* (2007) observed a reduction in the concentration of androstenedione in the culture medium of mouse ovarian cells treated with dexamethasone. This is interesting given that the thecal cells, which are not known to express GR, are responsible for producing androstenedione (Hsueh and Erickson, 1978; Tetsuka *et al.*, 1999; Van Merris *et al.*, 2007; Kirby *et al.*, 2009). Nonetheless, reductions in androstenedione production would be expected to correlate with decreased oestradiol concentrations, but this may only be relevant during periods of chronic stress as sufficient androstenedione molecules would be present to maintain oestradiol production for a time.

Glucocorticoids dose-dependently inhibit the production of oestradiol from bovine and rat granulosa cells treated with FSH and androstenedione *in vitro*, which suggests a direct pathway for the

suppression of oestradiol production (Hsueh and Erickson, 1978; Kawate *et al.*, 1993). It is likely that GC decrease oestradiol production by suppressing the activity and/or production of aromatase, the enzyme responsible for catalysing the conversion of androstenedione to oestradiol (Hsueh and Erickson, 1978). However, GC treatments do not appear to inhibit oestradiol production in rat granulosa cells that already exhibit high levels of aromatase activity (Hsueh and Erickson, 1978), indicating that GC likely act to disrupt the FSH-induced aromatase activity rather than directly suppressing aromatase enzymes (Hsueh and Erickson, 1978). Regardless of the pathway (direct or via decreased LH concentrations), it is clear that stress and the associated rise in GC concentrations have been linked to a decrease in the production of oestradiol.

Glucocorticoids have also been shown to dose-dependently inhibit the expression of LH-R within cultured bovine granulosa cells (Kawate *et al.*, 1993). The up-regulation of LH-R expression in ovarian granulosa cells is vital to priming the cells for the LH surge (Kawate *et al.*, 1993), thus the down-regulation of LH-R expression by GC would likely impede ovulation. An abundance of LH-R within granulosa cells is irrelevant if the pre-ovulatory LH surge is absent, thus in terms of ovulation, the effects of inhibitory GC on the pre-ovulatory LH surge are probably of greater significance. Despite this, reduced LH-R expression may disrupt ovulation by decreasing the responsiveness to LH surge.

Oocytes are kept in a state of meiotic arrest until the pre-ovulatory LH surge occurs, which enables meiosis to recommence (Yang *et al.*, 1999; Whirledge and Cidlowski, 2010). Yang *et al.* (1999) found that both dexamethasone and cortisol disrupt and delay the meiotic, but not cytosolic, maturation of cumulus enclosed pig oocytes *in vitro*. Glucocorticoids have been shown to reduce the activity of mitogen activated protein kinases (MAPK), which have an important role in the regulation of meiotic arrest and resumption of meiotic development following the LH surge or exogenous hormone treatments (Liang *et al.*, 2007; González *et al.*, 2010; Cui *et al.*, 2012). Low levels of MAPK activity have been shown to cause degeneration of the spindle assembly in rat oocytes *in vitro*, causing chromosomes to be dispersed around the ooplasm (Cui *et al.*, 2012). While the effect of GC on MAPK activity in oocytes is difficult to study directly *in vivo*, it is likely that high GC concentrations affect the nuclear and/or cytoplasmic maturation of oocytes via the inhibition of MAPK.

2.5 Domestic cats as a model for non-domestic felids

As mentioned in Chapter 1, most felids are considered threatened or endangered in part, if not all, of their natural home range. Consequently, studies involving the manipulation of these species need to be carefully designed to ensure that the welfare or conservation value of study animals is not adversely affected. The domestic cats has frequently been used as a model to develop and validate research methods prior to conducting research in non-domestic felids.

The reproductive physiology (e.g., oestrous and spermatogenic cycles) of domestic cats is comparable to that of non-domestic felids (Andrews *et al.*, 2019). In fact, reproductive physiology is fairly similar across Felidae, with the only exception being the prolonged NPLP of the *Lynx spp.* (Andrews *et al.*, 2019). In the context of reproductive biology, the domestic cat is widely considered to be a model species that is representative of Felidae. Indeed, the domestic cat has served as a starting point for much of the reproductive-based research on non-domestic felids, with numerous topics including the reproductive endocrinology, exogenous control of ovarian function, sperm collection, and ARTstudied in the domestic cats before being implemented or researched in non-domestic felids (Sojka *et al.*, 1970; Platz *et al.*, 1978; Shille *et al.*, 1983; Shille *et al.*, 1984; Goodrowe *et al.*, 1988a; Goodrowe *et al.*, 1988b; Howard *et al.*, 1990; Donoghue *et al.*, 1992; Howard *et al.*, 1992b; Pope *et al.*, 1993; Pelican *et al.*, 2003; Neubauer *et al.*, 2004).

The ability to use domestic cats as a model species has enabled researchers to conduct studies that would likely be deemed inappropriate in endangered felids. For example, it is difficult to study testicular or ovarian histology in living endangered felids as it requires gonadectomy and thus the loss of the animal for breeding purposes. As the breeding value of endangered felids is often too high to justify de-sexing, studies assessing testicular or ovarian histology (e.g., histomorphology, receptor expression, spermatogenic cycle, and apoptosis rates) are typically limited to the domestic cat model (Neubauer *et al.*, 2004; Müller *et al.*, 2012).

Another major challenge for research in non-domestic felids is animal availability and limited sample sizes. Research on non-domestic felids is often limited to small sample sizes (i.e., less than five individuals) or requires the use of animals from different conservation or research institutes (Roth *et al.*, 1994; Swanson *et al.*, 1996a; Moreira *et al.*, 2001; Newell-Fugate *et al.*, 2007; Gañán *et al.*, 2010; Lueders *et al.*, 2014). This approach can introduce error or variation due to confounding factors such as differences in management practices, diet, climate, or photoperiod. Domestic cat colonies allow researchers to use (a) larger sample sizes and (b) minimise variation in the environment and management of different animals.

2.6 Simulating the endocrine stress response in domestic cats

Glucocorticoids appear to be one of the main pathways by which stress suppresses the HPG axis in mammals (Figure 2.6; Appendix 4), thus it may be possible to study the effects of captivity-related stress on the ovarian and testicular function of felids by mimicking the elevated circulating GC concentrations observed in captive animals. As mentioned previously, captive cheetah, clouded leopards, and Canadian lynx have had faecal GC concentrations measured that are 2.5 times higher than their wild conspecifics (Wielebnowski *et al.*, 2002a; Terio *et al.*, 2004; Fanson *et al.*, 2012). An exogenous GC treatment (e.g., prednisolone or hydrocortisone) could be used induce a similar increase in circulating GC concentrations. Indeed, GC treatments have been used to study stress

physiology in other species such as mice and rats (Bambino and Hseuh, 1981; Orr and Mann, 1992; Chen *et al.*, 2012).

In domestic cats, basal serum cortisol concentrations range from 1.8 ng/mL to 58 ng/mL (Peterson *et al.*, 1994; Feldman and Nelson, 2004), and based on the upper limit of this range, serum GC concentrations of approximately 150 ng/mL would be equivalent to the increases observed in captive non-domestic felids. So theoretically, artificially maintaining serum GC concentrations at ~150 ng/mL in domestic cats using exogenous GC would simulate the elevated GC concentrations observed in captive non-domestic felids.

Prednisone is the main exogenous GC treatment used in small animal veterinary practice (Behrend and Kemppainen, 1997). Prednisone is biologically inactive and is activated through the conversion to prednisolone by 11β-hydroxysteroid dehydrogenase in the liver (Jenkins and Sampson, 1967). However, the conversion of prednisone to prednisolone is inefficient in domestic cats, with only 21% of oral prednisone being converted to prednisolone (Graham-Mize *et al.*, 2005). Consequently, oral prednisolone is the main exogenous GC treatment for cats.

In cats, prednisolone is given orally and typically used to treat and range of conditions including skin irritations, inflammation associated with joint disease (e.g., arthritis), some autoimmune conditions, and Addison's disease/hypoadrenocorticism. Prednisolone is thought to be an intermediate-acting GC, with a reported half-life of 12-36 hours (Behrend and Kemppainen, 1997; Feldman and Nelson, 2004). The physiological replacement dose (i.e., the dose required to replace cortisol concentrations in hypoadrenocorticism) of prednisolone in cats is estimated to be 0.44 mg/kg/day (Behrend and Kemppainen, 1997). The doses of prednisolone are divided into two categories: anti-inflammatory (1-2 mg/kg bodyweight per day) and immunosuppressant (2.2-8.8 mg kg bodyweight per day; Behrend and Kemppainen, 1997). Prednisolone acts via the same glucocorticoid receptors as endogenous cortisol, so the biological effects of prednisolone are similar to that of cortisol (Behrend and Kemppainen, 1997). However, prednisolone has a four to five times higher GC potency than endogenous cortisol or exogenous hydrocortisone (i.e., synthetic cortisol; Behrend and Kemppainen, 1997; Lowe *et al.*, 2008a).

While GC are used to treat a range of conditions, there are possible side effects of GC therapy, particularly with long-term administration in many species. Cats appear to tolerate GC treatments quite well, with reports of adverse side effects of GC therapy being relatively uncommon (Lowe *et al.*, 2008a). Indeed, a study on 15 cats found that the administration of prednisolone or dexamethasone at immunosuppressive doses for two months resulted in no clinical side effects except for mild polyuria and polydipsia (Lowe *et al.*, 2008a). However, the long-term administration of GC leads to the suppression of the endogenous HPA axis (since GC negatively feedback on all levels of the HPA

axis), which can be problematic following the cessation of the treatment, with cats displaying hypoadrenacorticism due to suppressed HPA activity (Middleton *et al.*, 1987). Therefore, it is important to taper cats off GC treatments rather than abruptly stopping the treatment. This allows the activity of the HPA axis to gradually increase prior to the complete removal of the treatment

2.7 Alternative methods for monitoring the ovarian function of felids

2.7.1 Accelerometry

As mentioned previously, the accurate and reliable monitoring of ovarian function is challenging for many felids due to a lack of overt oestrous behaviours (Asa *et al.*, 1992; Graham *et al.*, 1995; Foreman, 1997; Wielebnowski and Brown, 1998; Moreira *et al.*, 2001; Brown *et al.*, 2002). However, it is unlikely that oestrus is truly silent, but rather it is associated with subtle behavioural changes that are challenging to identify without extensive behavioural monitoring (Asa *et al.*, 1992; Graham *et al.*, 1995; Foreman, 1997; Wielebnowski and Brown, 1998; Moreira *et al.*, 2001; Brown *et al.*, 2002). Interestingly, many of the behaviours that felids express more frequency during oestrus appear to correlate with an increase in overall physical activity (OPA), so oestrus might be detectable by close monitoring of an animal's activity levels (Asa *et al.*, 1992; Graham *et al.*, 1995; Foreman, 1997; Wielebnowski and Brown, 1998; Moreira *et al.*, 2001; Brown *et al.*, 2002).

Accelerometry may offer a means of indirectly monitoring the activity and behaviour of felids. Indeed, Actical® 'Minimitter' Accelerometers have been attached to collars of domestic cats and validated as an accurate and automated means of assessing OPA, thus removing the need for continuous, time-consuming, and potentially intrusive direct behavioural observations (Lascelles *et al.*, 2008; Andrews *et al.*, 2015). The Actical® devices contain a single omnidirectional accelerometer; (Lascelles *et al.*, 2008). Andrews *et al.* (2015) validated Actical® accelerometers for cats by comparing observed activity and Actical® counts. While there was a strong correlation between the observed activity and Actical® counts of individual cats (mean ± SEM, r^2=0.94 ± 0.03), there was considerable variation in the activity of different cats and the linear regression between the observed activity and Actical® counts of different cats (Andrews, 2015).

Preliminary research suggests that accelerometry could be used to detect a measurable increase in OPA during behavioural oestrus in cats (Andrews, 2015). Accelerometry has been used to detect an increase in OPA with oestrus/follicular growth in a range of species including dairy cattle (*Bos taurus*; At-Taras and Spahr, 2001; McGowan *et al.*, 2007), mice (*Mus musculus*; Kopp *et al.*, 2006), rats (*Rattus norvegicus*; Gerall *et al.*, 1973), and pigs (*Sus scrofa*; Cornou, 2006). In dairy cattle, accelerometry was able to accurately to detect more than 90% of real oestrus events (At-Taras and Spahr, 2001; McGowan *et al.*, 2007). Thus, accelerometry may also offer a minimally invasive technique for monitoring the ovarian function of felids in felids, pending the automated analysis and interpretation of the activity data.

Heyrex® activity monitors contain a triaxial accelerometer and have been developed to provide an automated analysis of accelerometry data, with the devices providing information on the total amount of time spent active (i.e., OPA) and the amount of time spent exhibiting of a range of behaviours (sleeping, resting, walking, running; Edwards and Gibson, 2012; Mejia *et al.*, 2019). Furthermore, the Heyrex® devices may have advantages over similar devices and be more practical in captive management context as data can be wirelessly upload data onto an online server that can be accessed anywhere in the world (c.f., the Actical® accelerometers require removal and manual downloads of the data). The Heyrex® devices were designed for dogs and have not yet been validated in domestic cats. However, the modification and validation of the Heyrex® devices for monitoring the activity and behaviour of cats would be valuable.

2.7.2 Infrared thermography

An alternative method for monitoring reproductive state is infrared thermography. The elevated oestradiol concentrations associated with oestrus have been linked to increased blood flow to the vulvar, which, in turn, increases the superficial temperature of the perivulvar area (Simões *et al.*, 2014; Talukder *et al.*, 2014). In cattle, pigs and horses, infrared thermography has been used to accurately detect both follicular growth and ovulation based on changes in the perivulvar skin temperature, with the vulva temperature rising during proestrus and oestrus, then decreasing considerably at the time of ovulation (Stelletta *et al.*, 2013; Redaelli *et al.*, 2014; Simões *et al.*, 2014; Talukder *et al.*, 2014; Sakatani *et al.*, 2016; Radigonda *et al.*, 2017). A similar trend has been reported for dogs (*Canis familiaris*), although the differences in vulvar temperature changes were not statistically significant due to large standard errors, possibly related to the confounding factors (e.g., climatic conditions and physical activity) or a high degree of inter-individual variation (Olğaç *et al.*, 2017). The use of thermal imaging to assess reproductive status in felids has not yet been investigated, but is warranted given that, if successful, it would offer a non-invasive method for rapidly detecting oestrus.

However, a major limitation of thermography is that it is highly affected by a range of factors such as temperature, humidity, sunlight, wind, focal distance, and fur density (Kastberger and Stachl, 2003; Cilulko *et al.*, 2013; Rekant *et al.*, 2016; Silva *et al.*, 2017). The amount of physical activity of the subject prior to taking a thermographic image can also cause variation in the recorded temperatures (Simões *et al.*, 2014; Silva *et al.*, 2017). Thus, the use of set reference points (e.g. on gluteal or perianal temperature) is essential to account for some of the variation caused by environmental factors, with researchers monitoring the relative temperature of the perivulvar area and the reference point (Simões *et al.*, 2014). It is also important to minimise confounding variables by controlling the environment (particularly ambient temperature and humidity) in which the IR images are taken. Ultimately, careful experimental design is needed when using IR thermography for research.

2.8 Thesis aims and objectives

As indicated in Chapter 1, the overall aim of this thesis was to identify and evaluate some of the factors that influence the reproductive performance of felids in captivity, and the first objective was to conduct the comprehensive review of felid reproduction and is presented here in Chapter 2. Following the review of the literature, the following additional objectives were identified:

1. To determine the effects of GC administration (i.e., a simulated endocrine stress response) on the testicular functions of felids (Chapter 3), and on the ovarian response of domestic cats to exogenous gonadotrophin (Chapter 4), using the domestic cat as a model species. This is a priority because it directly tests the hypothesis that physiological stress can affect reproductive performance in felids.
2. To develop and validate Heyrex® accelerometers (designed for dogs) for monitoring the activity and behaviour cats (Chapter 5). This is important because the validation of these devices will provide a minimally-invasive and low-stress means of continuously monitoring the activity and behaviour of cats without the need for extensive and time consuming observational assessments.
3. To evaluate whether accelerometry (activity monitoring) and/or IR thermography (PVT monitoring) could be used to monitor the ovarian function of domestic cats, as a model for endangered felids (Chapter 6). Development of a reliable and low-stress (i.e., non-invasive or minimally invasive) method for monitoring the ovarian function of felids could potentially improve the captive management (e.g., determine when to combine breeding pairs), and thus, the success of captive felid breeding programs.
4. An overall synthesis of the thesis is presented in Chapter 7. This Chapter identifies key findings and presents recommendations for future research.

Chapter 3

The effects of a simulated endocrine stress response on testicular function of domestic cats (*Felis catus*)

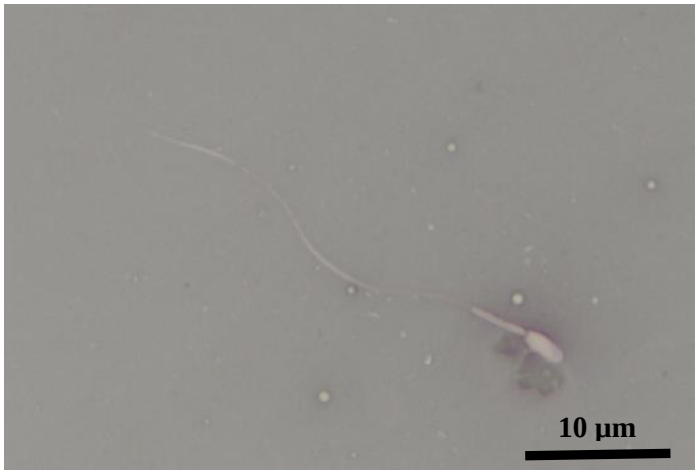

Photo of normal feline spermatozoa (1000 X magnification), by Chris Andrews.

Chapter 3: The effects of a simulated endocrine stress response on testicular function of domestic cats (*Felis catus*)

3.0 Abstract

Elevated glucocorticoid (GC) concentrations associated with captivity-related stress have been linked to impaired testicular function and sperm quality in felids, but putative physiological evidence is lacking. This study aimed to determine the effects of artificially elevated glucocorticoid concentrations on testicular function and sperm quality of felids using the domestic cat (*Felis catus*) as a model species. Sixteen intact male domestic cats aged from 1 – 13 years (2.43 ± 0.78 years) were randomly divided into treatment (n=8) and control (n=8) groups. Cats in the treatment group were given 1 mg/kg/day oral prednisolone (Redipred) for 50 days which encompassed an entire sperm cycle. Blood samples were taken on days 0, 2, 4, 7, 10, 20, 30, 40, 50 (prior to neutering) and 60 of the trial. All cats were orchiectomised on day 50, after which epididymal sperm samples were assessed and the testes fixed for histological assessment. Cortisol concentrations were lower in treatment cats (5.2 ± 0.9 ng/mL; mean ± SEM) than in control cats (15.1 ± 1.1 ng/mL, P<0.001). Testosterone concentrations did not differ between the two groups. While sperm motility was similar between the treatment and control groups, cats given prednisolone had a higher proportion of morphologically abnormal sperm in both the caput (72.5% vs. 59.6%, P<0.001) and cauda (56.7% vs. 35.8%, P<0.001) epididymis. Testicular histomorphometric data were similar between the control and treatment groups. The total number of germs cells per seminiferous tubule cross section did not differ between the two groups, nor did the relative abundance of spermatogonia, spermatocytes, and spermatids. Cats given prednisolone had fewer Sertoli cells per tubule cross-section than those in the control group (17.1±0.9 vs. 19.7±0.8, P=0.04). This was likely related to the greater number of apoptotic Sertoli cells per tubule cross-section in treatment vs. control cats (0.25 ± 0.02 vs. 0.10 ± 0.02 apoptotic Sertoli cells, P<0.001). Furthermore, Sertoli cell load (number of germ cells per Sertoli cell) was also higher in the treatment group (11.5 ± 0.8 vs. 9.4 ± 1.2, P<0.001) and was positively correlated with the percentage of morphologically abnormal sperm in the epididymis (r^2 = 0.78, P<0.001). In conclusion, the prednisolone treatment resulted in an increase in the proportion of morphologically abnormal sperm in the epididymis, and this may be related to an increase in Sertoli cell load. These findings provide quantitative evidence to support the previously untested hypothesis that elevated GC concentrations, such as those resulting from captivity-related stress, have the potential to impair testicular function and sperm quality in felids.

3.1 Introduction

Captive breeding programs are a vital component of conservation strategies for felids, but these programs are often hindered by poor reproductive performance (Mellen, 1991; Terio *et al.*, 2004;

Brown, 2006; Moreira *et al.*, 2007; Fanson *et al.*, 2010; Brown, 2011). A recent review on the reproductive biology of felids highlighted the high incidence of teratospermia (>60% morphologically abnormal sperm) as a major factor contributing to poor success of both natural and assisted breeding programs (Chapter 2; Andrews *et al.*, 2019).

Low levels of genetic diversity have been linked to teratospermia in felids (Wildt *et al.*, 1987a; Brown *et al.*, 1991; Pukazhenthi *et al.*, 2006b; Andrews *et al.*, 2019), although teratospermia is also prevalent in several species that exhibit reasonably high genetic diversity (Schwartz *et al.*, 2003; Swanson, 2003; Pukazhenthi *et al.*, 2006b; Schmidt *et al.*, 2011; Andrews *et al.*, 2019). Captivity-related stress (i.e., the activation of the hypothalamic-pituitary-adrenal axis in response to factors associated with the captive environment) may also contribute towards the high incidence of teratospermia in felids (Chapter 2). Several felid species including Canadian lynx (*Lynx Canadensis*), cheetah (*Acinonyx jubatus*), and clouded leopards (*Neofelis nebulosa*) have been found to exhibit higher basal faecal glucocorticoid (GC) concentrations (a physiological indicator of stress) in captivity than in the wild (Wielebnowski *et al.*, 2002a; Terio *et al.*, 2004; Fanson *et al.*, 2012).

Glucocorticoids act on all levels of the hypothalamic-pituitary-gonadal axis to supress testicular steroidogenesis and spermatogenesis (Orr and Mann, 1992; Sharpe *et al.*, 1992; Yazawa *et al.*, 2000; Gore *et al.*, 2006; Kirby *et al.*, 2009). Exogenous GC have been found to indirectly (via decreased gonadotrophin-releasing hormone or luteinising hormone (LH) production) and directly inhibit the production and secretion of testosterone in rats (Bambino and Hsueh, 1981; Welsh *et al.*, 1982; Whirledge and Cidlowski, 2010). The elevated GC concentrations observed in captive male felids have also been linked to decreased plasma testosterone concentrations (Terio *et al.*, 2004; Fanson *et al.*, 2012). Abnormally low testosterone concentrations have been found to disrupt late spermatogenesis, lead to poor germ cell-Sertoli cell adhesion, increase germ cell degeneration, and result in a higher proportion of morphologically abnormal sperm (Meistrich *et al.*, 1975b; Sharpe *et al.*, 1992; Walker, 2003; Mruk and Cheng, 2004).

The captive environment also appears to affect sperm quality or concentration in the ejaculates of some male felids including jaguars (*Panthera onca*), cheetah, and lions (*Panther leo*; Koester *et al.*, 2015; Andrews *et al.*, 2019). However, the ejaculates of captive and wild individuals have only be compared for five felid species (Chapter 2; Andrews *et al.*, 2019), and it is unclear whether any differences in the ejaculate traits are due to captivity-related stress or other factors associated with captivity (e.g., diet). Koester *et al*. (2015) found that the cheetah produce ejaculates with a higher concentration of motile sperm when held off-exhibit than when on-exhibit. Given that the daily management and diets of cheetah on and off exhibit were similar, it would suggest that the stress associated with public exhibition adversely affected sperm output in this species (Koester *et al.*,

2015). To date, however, no study has directly examined the underlying hypothesis that elevated GC levels, as might derive from captivity-related stress, actually impact negatively on male felid fertility.

Thus, this study aims to determine the effects of a simulated endocrine stress response (i.e., exogenous GC treatment) on testicular function and spermatogenesis of felids using the domestic cat (*Felis catus*) as an animal model.

3.2 Materials and methods

3.2.1 Animals

Sixteen healthy, intact male domestic cats that aged 2.43 ± 0.78 (range: 1.37 – 14.02) years and weighed 4.18 ± 0.12 (range: 3.24 – 4.67) kg were used for this trial. The cats were housed in a purpose-built colony cage at the Centre for Feline Nutrition, Massey University, Palmerston North, New Zealand (175◦3'E, latitude 40°22'S, longitude) in groups of eight. The cats were fed a complete and balanced (AAFCO, 2020) commercial moist (canned) feline diet (Kraft Heinz Wattie's Ltd., Hastings, New Zealand) with *ad libitum* access to water. The husbandry of the cats complied with the Animal Welfare (Cats) Code of Welfare (Anonymous, 2007) and all research was conducted in accordance with MUAEC protocol number 19/09.

3.2.2 Experimental design

The cats were allocated randomly (using www.randomizer.org) into a treatment group (*n*=8) and a control group (*n*=8). For logistic reasons, the trial was conducted in two replicates, with four control and four treatment cats per replicate. Cats in the treatment group were given 1 mg/kg oral prednisolone (Redipred: Aspen Pharmacare Australia Pty. Ltd., Saint Leonards, NSW, Australia) daily for 50 days. The 50-day treatment period was selected to encompass the entire spermatogenic cycle of cats, which takes approximately 47 days (França and Godinho, 2003). In cats, the effective cortisol replacement dose using prednisolone has been estimated to be 0.44 mg/kg/day (França and Godinho, 2003). The administration of 1 mg/kg/day prednisolone was selected as it would be comparable to the elevated cortisol concentrations associated with captivity-related stress, estimated to be 2.0 – 2.5 times higher than basal concentrations (Wielebnowski *et al.*, 2002a; Terio *et al.*, 2004; Fanson *et al.*, 2010).

Jugular venepuncture was used to collect 2 mL blood samples from all cats on Days 0 (immediately before first GC treatment), 2, 4, 7, 10, 20, 30, 40, and 50 (prior to neutering) of the trial. Local anaesthetic (Emla cream, 5% lignocaine; Aspen Pharmacare Australia Pty Ltd., Saint Leonards, NSW, Australia) was applied to the skin of the neck 15-30 min prior to blood collection. Blood samples were collected into vacuum tubes (Becton and Dickinson Co., Franklin Lakes, NJ, USA), left for 2-3 hours at room temperature, and then centrifuged at 2500 rpm for 10 minutes and the serum extracted. Serum samples were stored at -80ºC until assayed.

On Day 50, all cats were given a single intramuscular injection of 0.05 mg/kg medetomidine (Dexdomitor: Zoetis Ltd., Auckland, New Zealand). After 10 minutes, an intramuscular injection of 5.0 mg/kg ketamine (Phoenix Pharm Distributors Ltd., Auckland, New Zealand) was administered. Once anaesthetised, the cats were castrated and both testes collected. Following orchiectomy, the cats were given an intramuscular injection of 0.2 mg/kg butorphanol (Butorgesic; Indivior Pty. Ltd., Macquarie Park, NSW, Australia), and a subcutaneous injection of 0.1 mg/kg meloxicam (Metacam: Boehringer Ingelheim, Auckland, New Zealand) for post-surgical analgesia. Lastly, the cats were given 0.025 mg/kg atipamezole for medetomidine reversal (Atipam: Jurox Pty Ltd., Rutherford, NSW, Australia) and monitored closely during recovery from anaesthesia. The cats were then administered 0.5 mg/mL meloxicam orally once a day for two to three days following castration. The cats in the treatment group were gradually tapered off the oral prednisolone, initially receiving a reduced dose of 0.5 mg/kg daily for six days following castration (Day 51-57), then 0.5 mg/kg every second day for a further six days (Days 58-64).

3.2.4 Epididymal sperm recovery and assessment

Immediately after orchiectomy, each testis was placed in 37ºC phosphate buffered saline (PBS; 72 mOsm and pH 7.2) with 5% foetal bovine serum (FBS; Life Sciences NZ Ltd, Auckland, New Zealand) and the epididymis was carefully dissected from the testis. The caput and cauda regions of the epididymis were then separated, and each placed into 300 µL of 37ºC PBS containing 5% FBS and macerated using a scalpel blade. The macerated epididymal sections were placed in a 37ºC incubator for 20 minutes. For each region, a 10 µL aliquot of the sperm suspension was used to assess the percentage of motile sperm and progressive motility graded in a scale of 0 - 5 (where 0 = no forward progression, and 5 = rapid forward progression) as previously described (Neubauer *et al.*, 2004; Müller *et al.*, 2012). The sperm motility index (SMI) was then calculated using the following equation: SMI = (% motile sperm + (20*progressive motility))/2 (Morato *et al.*, 2001; Crosier *et al.*, 2009; Gañán *et al.*, 2010). For each region, a 30 µL aliquot of the sperm suspension was stained with 10 µL Eosin-Nigrosin (Minitüb GmbH, Tiefenbach, Germany) and compound microscopy (1000 X magnification) used to assess the morphology of 100 sperms as described previously (Müller *et al.*, 2012).

3.2.5 Testis tissue processing

Testes were weighed (gross testicular weight) immediately after removal of the epididymides (Neubauer *et al.*, 2004; Müller *et al.*, 2012). The density of cat testicular tissue is 1.02 (França and Godinho, 2003), thus testicular volume was considered to be equal to testis weight. Net testicular weight was calculated by subtracting 19% of gross testicular weight to account for the weight of the tunica albuginea (França and Godinho, 2003). The gross weights of the two testes for each cat were combined and the gonadosomatic index (GSI; %) calculated as: (testicular mass [g]/bodyweight [g])*100 (Müller *et al.*, 2012). Testes were placed in 4% paraformaldehyde in PBS for 24 hours, cut

in half transversely and returned to the formalin solution for one week, then embedded in paraffin wax.

3.2.6 Histomorphology

Sections of testicular tissue were cut at 4 µm thickness, placed on slides, and dried at room temperature. The slides were deparaffinised by immersion in xylene for 5 minutes, washed in 100% ethanol for 5 minutes, rehydrated by sequential immersion in ethanol (95%, 85%, 70%, and 50%), and stained with haematoxylin and eosin using a Leica autostainer XL (Leica Biosystems, Wetzlar, Germany). The slides were permanently mounted using the Leica CV5030 (Leica Biosystems, Wetzlar, Germany) coverslipper using Entellan (Merck Group, Darmstadt, Germany) as the mounting medium. Images of 100 seminiferous tubules were taken from each testis using an Olympus BX 51 microscope, Olympus SC100 camera, and the Olympus CellSens imaging software (Olympus Corporation, Tokyo, Japan).

3.6.1 Volume densities

The volume densities (%) of the various tubular and inter-tubular components of the testis were determined using point-counting (França and Godinho, 2003; Neubauer *et al.*, 2004; Müller *et al.*, 2012). Point-counting was conducted using a 450-intersection grid, which was superimposed on images taken at 400 X magnification using ImageJ Version 1.52a (National Institutes of Health, Bethesda, MD, USA). Ten images were assessed per cat (five images per testis), thus 4500 intersection points were assessed per animal. Intersection points were classified as follows: seminiferous tubule components - tunica propria, seminiferous epithelium, or lumen; inter-tubular components - Leydig cells, connective tissue, blood vessels or lymphatic tissue (França and Godinho, 2003). The absolute volume densities (µL) of each of the testicular components were estimated using the following equation: absolute volume of testicular component [µL] = volume density of the testicular component [%]*net testicular weight [mg] (França and Godinho, 2003; Müller *et al.*, 2012).

3.2.6.2 Seminiferous tubule morphometrics

For each testis, the morphology of 50 circular seminiferous tubules was assessed using a light microscope (200-400 X magnification). The tubular diameter (average of two perpendicular measurements) and epithelial height (average of four measurements from the basement membrane to the lumen) were measured for each tubule cross-section (Figure 3.1). The total seminiferous tubule length per testis was determined as previously described by Müller *et al.* (2012), with total seminiferous tubule length [µm] = seminiferous tubule absolute volume [μm^3]/π(seminiferous tubule radius [$\mu m]^2$).

3.2.6.3 Sertoli and germ cell assessments

The total number of Sertoli cells, spermatogonia, spermatocytes, round spermatids, and elongate spermatids were determined for a total of 25 seminiferous tubule cross-sections per testis (Figure 3.1; França and Godinho, 2003; Müller *et al.*, 2012). The relative percentages of the different germs cell

types were calculated for each testis. The number of Sertoli cells per testis and per gram of testis were calculated from the number of Sertoli cell counts per tubule cross-section and the total seminiferous tubule length (França and Godinho, 2003; Müller *et al.*, 2012). The total counts of Sertoli cells, spermatogonia, spermatocytes, round spermatids, and elongate spermatids (for all 25 tubules) were used to calculate the total number of germ cells per Sertoli cell (i.e., Sertoli cell load) and the number of spermatids per Sertoli cell for each testis.

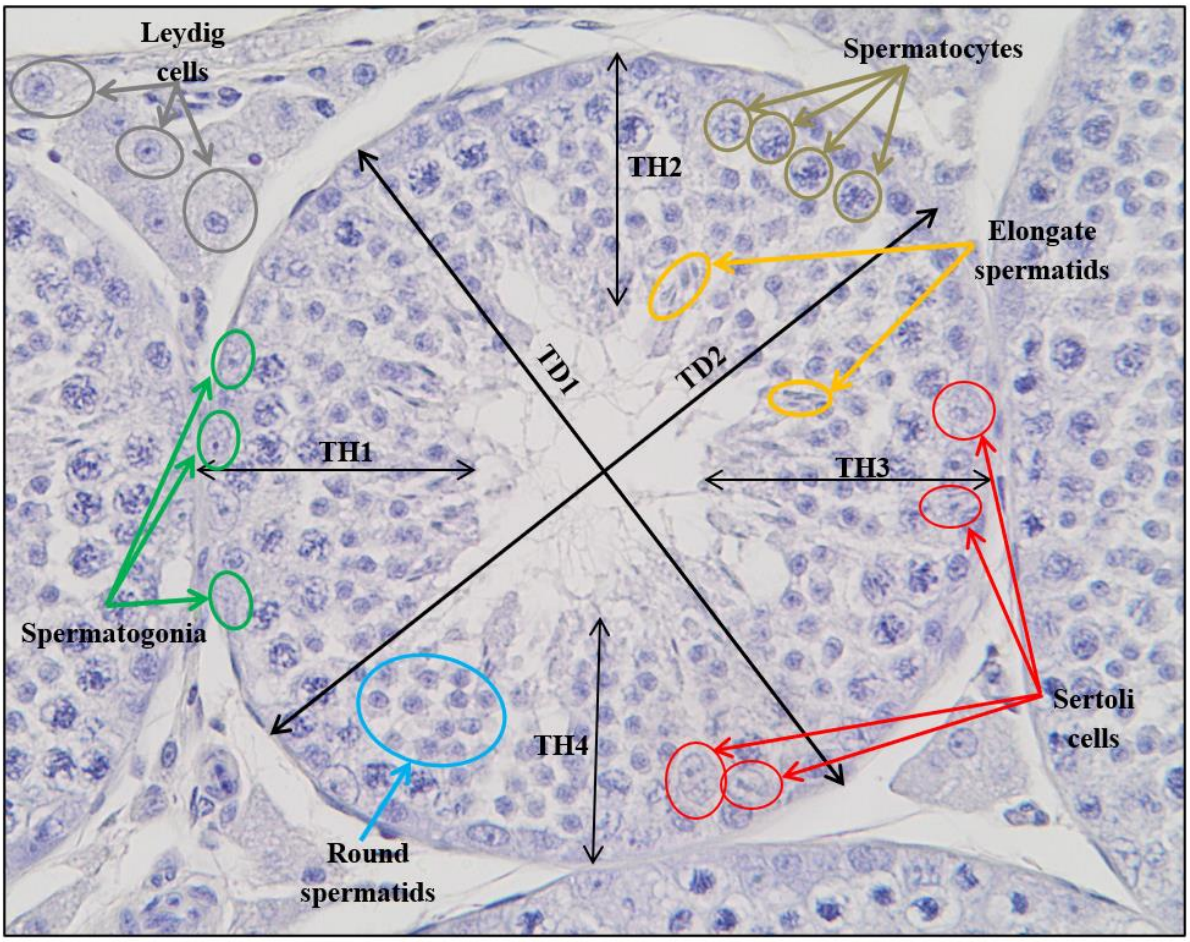

Figure 3.1 Seminiferous tubule of the domestic cat showing the different cell types present and the measurements carried out. Tubule diameter (average of TD1 and TD2) and seminiferous tubule height (average of TH1, TH2, TH3, and TH4), Leydig cells (grey), Sertoli cells (red), spermatogonia (green), spermatocytes (brown), round spermatids (blue), and elongate spermatids (yellow).

3.2.7 Determination of Apoptosis using TUNEL assay

The detection of apoptotic cells (i.e., those with fragmented DNA) in testicular sections was performed using the DeadEnd™ Colorimetric TUNEL system (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions.

Fifteen of the 30 collected testes (i.e., one testis per cat) were sectioned and assessed for apoptosis. Small intestine tissue from chicken was used as a positive control. For an additional positive control, a sections of cat testes and chicken small intestine were treated with 100 µL of 1 mg/mL bovine pancreatic deoxyribonuclease I recombinant (DNase; Roche Holding AG, Basel, Switzerland) in DNase buffer (40 mM Tris-HCl with pH 7.9, 10mM NaCl, 6mM $MgCl_2$, and 10mM $CaCl_2$) for 10 minutes to induce DNA fragmentation. Another section of cat testis and chicken small intestine tissue were used as a negative control, in which the recombinant terminal deoxynucleotidyl transferase (rTdT) enzyme was omitted from the TUNEL assay protocol below.

All tissue processing hereafter was conducted at room temperature unless otherwise stated. The slides were deparaffinised by immersion in xylene for 5 minutes and rehydrated by sequential (three minutes per concentration) immersion in ethanol (95%, 85%, 70%, and 50%). The slides were then washed in 0.85% NaCl in deionised water for 5 minutes and then PBS for 5 minutes. The slides were re-fixed in 10% buffered formalin in PBS for 15 minutes and subsequently washed twice with PBS (5 minutes per time). Excess liquid was removed and tissue on each slide was covered with 100 µL of 20 µg/mL proteinase K solution in proteinase K buffer (100 mM Tris-HCl and 50 mM ethylenediaminetetraacetic acid; pH 8.0) for 20 minutes to permeabilise the tissue. Sections were then washed in PBS for 5 minutes, re-fixed in 10% buffered formalin for 5 minutes, and rinsed in PBS two times (5 minutes per time).

Sections were incubated for 10 minutes with 100 µL of equilibration buffer (provided in the kit). Excess equilibration buffer was blotted off and the tissue sections were covered with 100 µL of the rTdT reaction mix (98 µL equilibration buffer, 1 µL biotinylated nucleotide mix, and 1 µL rTdT), before a plastic cover slip was placed on each slide and the sections incubated for 60 minutes at 37ºC in a humidified chamber. During incubation with the rTdT reaction mix, the biotinylate nucleotides were incorporated into the 3'-OH DNA ends of apoptotic cells by the rTdT enzyme. After incubation with the rTdT reaction mix, the plastic cover slips were removed, and the end-labelling reaction stopped by rinsing the sections in a Coplin jar containing saline sodium citrate buffer (SSC) for 15 minutes. Sections were then washed in PBS three times (5 minutes per time) to remove excess biotinylated nucleotides.

Endogenous peroxidases were blocked by immersing the sections in 0.3% hydrogen peroxide in PBS for 5 minutes, and then washed three times in PBS (5 minutes per time). Subsequently, sections were immersed in 100 µL of 1 µg/mL streptavidin – horseradish peroxidase in PBS for 15 minutes to allow streptavidin to bind to the biotin of the biotinylated nucleotides. The sections were then washed in PBS three times (5 minutes per time) and then covered with Betazoid 3,3′-Diaminobenzidine (DAB) Chromogen solution (Biocare Ltd., Concord, CA, USA) for 10 minutes. The horseradish peroxidase bound to the streptavidin-biotinylated nucleotide complexes catalysed the oxidation of DAB by

hydrogen peroxide, thus forming a brown precipitate/stain that could be observed using light microscopy. Lastly, the DAB solution was rinsed off with deionised water and the slides counterstained with haematoxylin and permanently mounted using Entellan as the mounting medium.

TUNEL-labelled tissue sections were examined at 400 X magnification. At total of 100 round seminiferous tubule cross-sections were assessed per testis and the number of TUNEL-labelled Sertoli cells, spermatogonia, spermatocyte, and round spermatids per cross-section were recorded. The first three testis slides were assessed twice to ensure consistency. Note that elongate spermatids were not assessed as the DAB staining was difficult to interpret in these cells.

3.2.8 Endocrine assays

3.2.8.1 Cortisol

Serum cortisol concentrations were analysed commercially by IDEXX Laboratories (Palmerston North, New Zealand). Cortisol concentrations were analysed using the Immulite® 1000 cortisol immunoassay. Cross-reactivity of the assay was reported as 100.0% for cortisol, 49.0% for prednisolone, 21.0% for methylprednisolone, 8.6% for corticosterone, 5.9% prednisone, and <1.0% for all other tested steroids (*n*=21). The analytical range was 2.0 – 500.0 ng/mL, and all samples and standards were run in duplicate. The mean intra-assay and inter-assay coefficients of variance (CV) were 7.13 ± 0.45% and 7.88 ± 0.51%, respectively.

3.2.8.2 Glucose

Glucose concentrations were used as an indicator for the efficacy of the prednisolone treatment and were analysed commercially by IDEXX Laboratories (Palmerston North, New Zealand). Serum glucose concentrations were determined using an enzymatic ultraviolet test (Beckman Coulter inc., Brea, CA, USA) and an AU680 clinical chemistry analyser (Beckman Coulter inc., Brea, CA, USA). The analytical range was 10.0 – 800.0 mg/dL. The mean intra-assay and inter-assay CV were 0.58 ± 0.06% and 1.11 ± 0.08%, respectively.

3.2.8.3 Prednisolone

Prednisolone, prednisone, cortisone, and cortisol were measured in the serum samples collected on Days 0, 10, 30, and 50 using ultra-high performance liquid chromatography (UHPLC) with mass spectrometry. The internal standard was cortisol-d4 (60 ng/mL), with 100 µL of the internal standard (in water) being added to 200 µL of blank plasma, standard (standards were 0.25, 0.63, 2.50, 7.50, 25.00, 75.00, 250.0, and 500.0 ng/mL for each steroid), quality control (QC), and collected serum samples.

Steroids were extracted by adding 1 mL of ethyl acetate (Merck KGaA, Darnstadt, Germany) to each tube and vortexing for 20 seconds followed by centrifugation (~2000 rpm for 5 minutes). The organic layer was removed to a new tube and dried. The residue was then resuspended in 60 µL of 35% methanol (Merck KGaA, Darnstadt, Germany) and 65% water and transferred to HPLC injector vials.

Ten µL was injected into a UHPLC mass spectrometer system consisting of an Accela MS pump and autosampler followed by an Ion Max APCI source on a Finnigan TSQ Quantum Ultra AM triple quadrupole mass spectrometer all controlled by Finnigan Xcalibur software (Thermo Electron Corporation, San Jose, CA, USA).

Steroid separation was achieved during a 14 minute run using a gradient of increasing methanol concentrations in water from 35 to 90% (gradient profile – 0.0 minutes = water 65%: methanol 35%, 5.0 minutes = 65%: 35%, 8.0 minutes = 10%:90%, 9.9 minutes = 10%:90%, 10.0 minutes = 65%:35%, and 14.0 minutes = 65%:35%), flowing at 400 µL/minute through a Kinetex F5 2.6 µm C18 100A 100 x 2.1 mm column (Phenomenex, Auckland, New Zealand) at 40ºC. Retention times were: prednisone 4.40 minutes, cortisone 4.75 minutes, cortisol 5.32 minutes, prednisolone 5.26 minutes, and cortisol-d4 5.28 minutes (Figure 3.2). Ionization was in positive mode, Q2 had 1.2 mTorr of argon for all steroids with the collision cell voltage between 22 and 28 volts. The mass transitions followed were: prednisone (359.2 → 237.1), cortisone (361.1 → 163.1), cortisol (363.2 → 122.2), prednisolone (361.2→147.2) and cortisol d4 (367.2 →121.2).

Analysis was carried out using Xcalibur™ software (Thermo Electron Corporation, San Jose, CA, USA). Steroid concentrations were calculated from the peak area ratio steroid/internal standard compared with standard curves dissolved in charcoal stripped human plasma (standards were 0.25, 0.63, 2.50, 7.50, 25.00, 75.00, 250.0, and 500.0 ng/mL for each steroid and generated from pure compounds).

3.2.8.4 Testosterone

Serum testosterone concentrations were measured using the Cobas® e601 analyser (Hitachi Ltd., Tokyo, Japan) and a commercially available electrochemiluminescence immunoassay (ECLIA), Elecsys Testosterone II Cobas® (Roche Diagnostics New Zealand, Auckland, New Zealand). Cross-reactivity was reported to be ≤18.0% for 11-β-Hydroxy-testosterone, ≤6.0% for 19-Northisterone, ≤3.2% for 11-Keto-testosterone, ≤2.5% for androstenedione, ≤2.4% testosterone proprionate, ≤2.4% ethisterone, ≤2.1% 5-α-Androstane-3β,17β-diol, and ≤1.0% for all other tested steroids (*n*=14).The detection range was 0.03 – 15.00 ng/mL. Values below the detection threshold were considered to be 0.0 ng/mL. The mean intra-assay CV for quality controls was 2.6%.

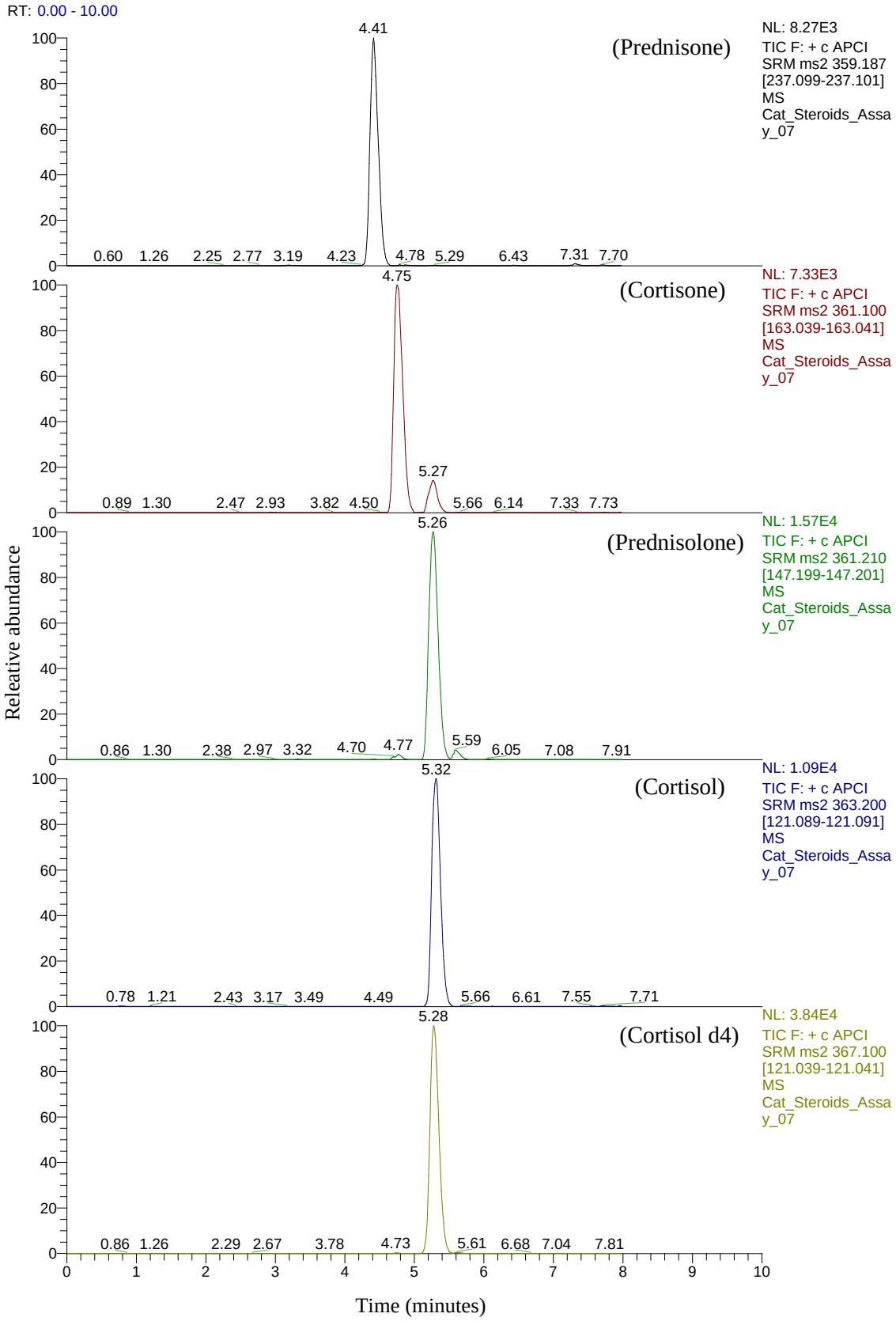

Figure 3.2 Chromatograms showing the retention times for prednisone, cortisone, prednisolone, cortisol, and cortisol d4 (i.e., the internal standard) as determined using ultra-high performance liquid chromatography with mass spectrometry. Abbreviations: atmospheric pressure chemical ionisation (APCI), mass spectrometry (MS), normalisation level (NL), selected reaction monitoring (SRM), tandem mass spectrometry (ms2), total ion chromatogram (TIC).

3.2.9 Statistical Analysis

RStudio version 1.0.143 (R Foundation for Statistical Computing, Vienna, Austria) and a significance level of $P<0.05$ was used for all statistical assessments. A trend or tendency was defined as $P<0.1$. Shapiro-Wilk normality test was used to assess the normality of all data. An effect of cohort was checked for all statistical comparisons between the control and treatment cats. When an effect of cohort was observed, a general linear model (GLM) was used to assess the effect of both treatment and cohort. The effects of epididymal region and treatment on sperm morphology were examined using multiple two-way ANOVA and Tukey's post hoc tests. The motility and morphology data of control and treatment cats were analysed using a Mann-Whitney-Wilcox test or Welch's t-test, depending on the normality of the data. The histomorphometric data of the control and treatments groups were compared using either a Mann-Whitney-Wilcox test or Welch's t-test. Mixed linear models were used to examine the effect of treatment and time (day of trial) on parametric endocrine data. When required, a power transformation (using the 'transformTukey' function in 'rcompanion') was used to normalise non-parametric data for the two-way ANOVA or mixed linear models. Correlations were assessed using either a Pearson's correlation coefficient (parametric) or Spearman ranked correlation coefficient (non-parametric).

3.3 Results

The mean body weight did not differ between the control (4.08 ± 0.18 kg) and treatment (4.14 ± 0.21 kg) groups ($P=0.85$). The bodyweights of the cats did not change significantly over the trial (mean change in bodyweight of -0.03 ± 0.04 kg). No adverse effects of the prednisolone treatment were observed in the treatment cats. However, one cat in the control group was diagnosed with feline infectious peritonitis and had to be euthanised prior to completion of this study, so all data from this cat were excluded from analysis.

3.3.1 Endocrine profiles

Pre-treatment (Day 0) serum cortisol, glucose, and testosterone concentrations did not differ between control and treatment groups; however, mean cortisol concentration from Day 2 – Day 50 differed significantly between groups (Table 3.1). When analysed overtime, by Day 2 cortisol concentrations were lower in the treatment group ($P=0.03$) and remained lower ($P<0.05$) on Days 4, 7, 10, 20, and 30 of the study (Figure 3.3). Cortisol concentrations decreased in the control group by Day 40, thus cortisol concentrations were not different between control and treatment cats, and they did not differ thereafter (Figure 3.3). On Day 60 (10 days after orchiectomy), cortisol concentrations were comparable to pre-treatment (Day 0) levels (Figure 3.3). Prednisolone and prednisone were undetectable in all serum samples.

Table 3.1 Mean (± SEM) serum cortisol, glucose, prednisolone, prednisone, and testosterone concentrations in untreated control cats (*n*=7) and treatment cats (*n*=8), given 1 mg/kg prednisolone for 50 Days. All cats were neutered on Day 50. Baseline samples were taken immediately before the first prednisolone treatment on Day 0. Treatment samples were taken from Day 2-50. ND = not detectable. NS = non-significant (*P*>0.10).

	Control	**Treatment**	**P-Value**
Baseline (Day 0)			
Cortisol (ng/mL)	18.2 ± 2.6	23.1 ± 6.6	NS
Prednisolone	ND	ND	-
Prednisone	ND	ND	-
Glucose (mg/dL)	73.6 ± 2.6	81.3 ± 3.3	0.09
Testosterone (ng/mL)	1.4 ± 0.6	2.1 ± 0.4	NS
Treatment (Mean of Day 2 – 50)			
Cortisol (ng/mL)	15.1 ± 1.1	5.2 ± 0.9	<0.001
Prednisolone	ND	ND	-
Prednisone	ND	ND	-
Glucose (mg/dL)	79.0 ± 1.8	85.2 ± 1.1	0.003
Testosterone (ng/mL)	2.3 ± 0.3	2.6 ± 0.2	NS

Glucose concentrations did not differ significantly over time (Figure 3.3). The mean glucose concentrations from Day 2 to 50 appeared to be higher in the treatment group than in the control group (*P*=0.003; Table 3.1), but glucose concentrations were not significantly different between the treatment groups on any given day (Figure 3.3).

Mean testosterone concentrations (2.3 ± 0.2 ng/mL, range: 0.0 – 7.5 ng/mL) did not differ between the control and treatment groups throughout the study (Table 3.1; Figure 3.3), thus they were combined for further assessment. Testosterone concentrations did not differ significantly over time, with the exception of Day 60 (Figure 3.3). Testosterone concentrations fell sharply after orchiectomy and were undetectable on Day 60 (i.e., 10 days after orchiectomy; Figure 3.3).

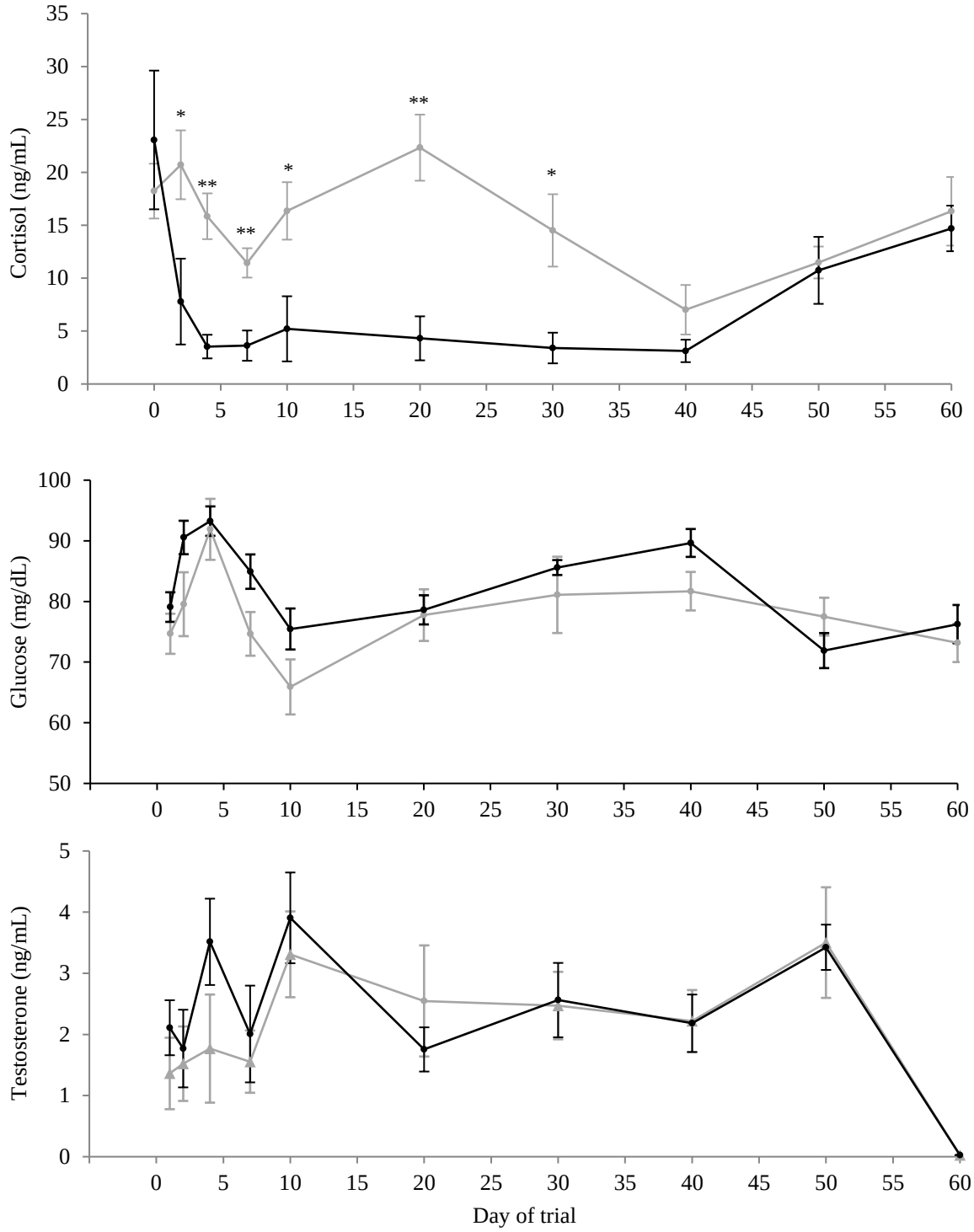

Figure 3.3 Mean (± SEM) serum cortisol (a), glucose (b), and testosterone (c) concentrations of untreated control cats (grey line; *n*=7) and treatment cats (black lines; *n*=8), which were given 1 mg/kg prednisolone for 50 days. All cats were neutered on Day 50. Cortisol concentrations differed between the control and treatment cats ($P<0.001$), and also differ significantly over time (excluding day 60) within group. There was no treatment by time effect for either glucose or testosterone, thus treatment and control groups were also compared for each day. *$P<0.05$ and **$P<0.01$.

3.2.2 Epididymal sperm assessment

Sperm motility, progressive motility, and SMI did not differ between the control and treatment groups, even when the caput and cauda regions were assessed independently. When considered independently of treatment group, the percentage of motile sperm and SMI values increased from the caput compared to the cauda epididymis (P<0.001 and P<0.001, respectively; Figure 3.4). Progressive motility was also lower in the caput than cauda epididymis (P<0.001).

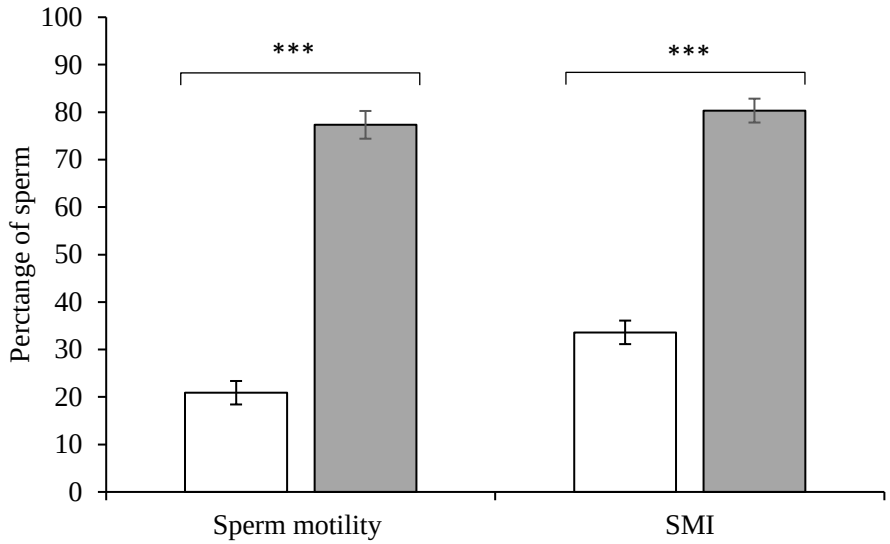

Figure 3.4 Mean (± SEM) sperm motility and sperm motility index (SMI) of sperm collected from the caput (white bars; n=15) and cauda (grey bars; n=15) epididymides of all cats (note: sperm motility and SMI did not differ between the control and prednisolone-treated cats). ***P<0.001.

The main sperm morphologies identified in this study are depicted in Figure 3.5. The percentage of morphologically abnormal sperm was higher in the treatment group than the control group (P<0.001), as was the percentage of both primary and secondary abnormalities (P<0.001 and P=0.01, respectively; Figure 3.6; Table 3.2). Total morphological abnormalities and primary abnormalities were significantly higher in the treatment group than the control group in both the caput and cauda epididymis (Figure 3.6; Table 3.2). Secondary abnormalities were also more prevalent in the caput (P<0.02) and cauda (P<0.001) epididymis of cats given prednisolone (Figure 3.6; Table 3.2).

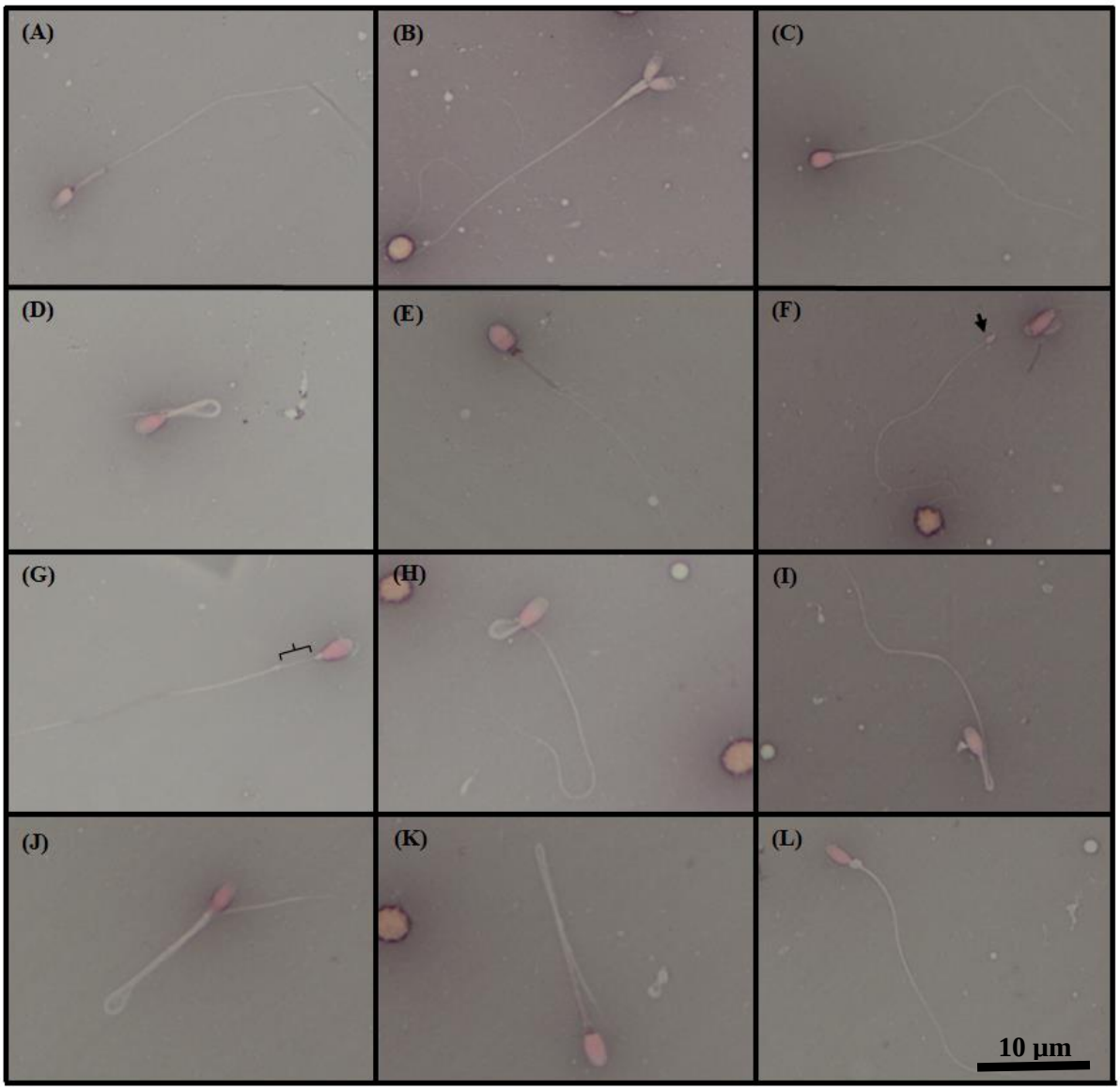

Figure 3.5 A summary of the sperm morphologies observed in epididymis of the domestic cat (1000 X magnification). (A) Normal morphology, (B) polycephalic, (C) polyflagellate, (D) coiled tail, (E) macrocephalic, (F) microcephalic (head indicated by arrow), (G) mid-piece aplasia, (H) bent mid-piece with cytoplasmic droplet, (I) bent mid-piece, (J) bent tail with cytoplasmic droplet, (K) bent tail, (L) proximal cytoplasmic droplet.

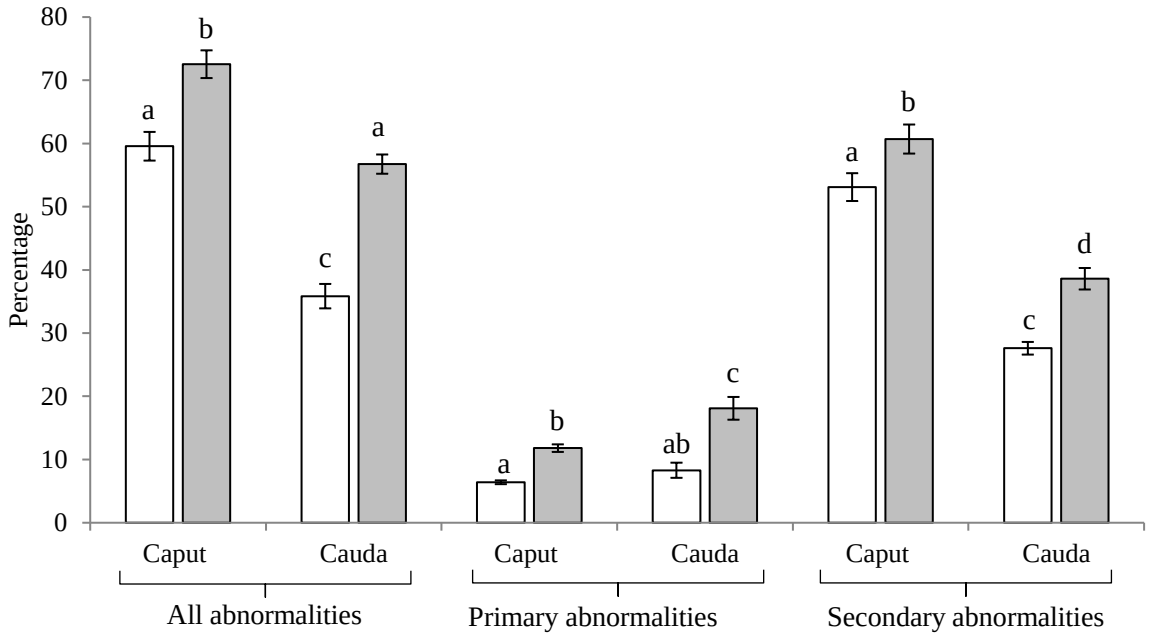

Figure 3.6 Mean (± SEM) percentage of all morphological abnormalities, primary abnormalities, and secondary abnormalities in the caput and cauda regions of the epididymides of untreated control (white bars; *n*=7) cats and cats treated with prednisolone (1 mg/kg) daily for 50 days (treatment, *n*=8, grey bars). Statistical comparisons (between treatment group and region) were done independently for all abnormalities, primary abnormalities, and secondary abnormalities using Tukey's Post hoc tests (parametric: morphological assessments). Statistical significance ($P<0.05$) is indicated by differing letters.

Table 3.2 Sperm traits for the caput and cauda epididymal regions of untreated control cats (*n*=7) and cats that received 1 mg/kg prednisolone daily for 50 days (*n*=8). Values presented as mean ± SEM. Statistical comparisons were made between the control and treatment cats in the caput and cauda regions independently. $^{\dagger}P<0.10$, * $P<0.05$, ** P<0.01, ***P<0.001.

	Caput		**Cauda**	
	Control	**Treatment**	**Control**	**Treatment**
Percentage motile (%)	22.5 ± 2.6	19.5 ± 2.4	76.7 ± 3.4	77.8 ± 2.5
Progressive motility (1-5)	2.5 ± 0.1	2.1 ± 0.2	4.4 ± 0.1	4.2 ± 0.1
Sperm motility index (%)	36.6 ± 2.1	31.0 ± 2.7	79.8 ± 2.9	80.8 ± 2.2
Abnormal sperm (%)	59.6 ± 2.3	72.5 ± 2.3***	35.8 ± 1.5	56.7 ± 1.5***
Primary sperm defects (%)	6.4 ± 0.3	11.8 ± 0.6***	8.3 ± 1.2	18.1 ± 1.8***
Acrosome defects	0.9 ± 0.5	1.7 ± 0.3*	0.5 ± 0.2	1.9 ± 0.3***
Head defects (Inc. Micro/macrocephalic)	3.0 ± 0.5	5.6 ± 0.9*	0.7 ± 0.2	2.9 ± 0.8*
Midpiece defects	1.8 ± 0.4	5.6 ± 0.9***	2.4 ± 0.4	7.8 ± 1.2***
Polycephalic	0.7 ± 0.2	1.0 ± 0.4	0.7 ± 0.3	1.9 ± 0.6†
Polyflagellate	0.1 ± 0.1	0.4 ± 0.2	0.4 ± 0.3	0.4 ± 0.2
Tightly coiled flagellum	2.3 ± 0.5	3.2 ± 0.6	7.0 ± 1.6	15.1 ± 2.2**
Secondary sperm defects (%)	53.1 ± 2.2	60.7 ± 2.3*	27.6 ± 1.0	38.6 ± 1.7***
Bent midpiece with droplet	1.7 ± 0.4	3.1 ± 0.6†	2.9 ± 0.8	5.2 ± 0.6**
Bent midpiece without droplet	2.1 ± 0.4	3.4 ± 0.5	3.4 ± 0.7	7.3 ± 1.1
Bent flagellum with droplet	0.6 ± 0.2	0.8 ± 0.3	1.7 ± 0.4	2.1 ± 0.6
Bent flagellum without droplet	0.8 ± 0.2	1.6 ± 0.6	2.9 ± 0.6	4.8 ± 1.0
Detached head	2.6 ± 0.7	4.5 ± 1.0†	4.4 ± 1.1	7.8 ± 1.0*
Distal cytoplasmic droplet	4.6 ± 0.9	10.1 ± 2.1**	12.5 ± 1.0	14.1 ± 2.6
Proximal cytoplasmic droplet	59.8 ± 3.4	64.2 ± 5.5*	11.3 ± 1.1	24.4 ± 3.2**
Spermatid	0.4 ± 0.2	0.9 ± 0.3	0.1 ± 0.1	1.1 ± 0.3***

3.2.3 Testicular histomorphometric parameters

The average testicular weight was 1.66 ± 0.08 g (range: 1.23 – 2.85 g), and there were no differences between left and right testis within cat. As such, data from the two testes of each cat were combined for further analysis. Body weights and combined testes weight were not correlated (*P*=0.21). While the average body weight and testicular weights of the cats did not differ significantly between the control and treatment groups, there was a tendency (*P*=0.08) for testis weight to be higher in the treatment group (Table 3.3). The GSI also did not differ between the treatment groups (Table 3.3).

Table 3.3 Testis histomorphometric data from cats in the control (*n*=7) and cats treated with 1 mg/kg prednisolone for 50 days (*n*=8). Seminiferous tubule length was calculated as: total seminiferous tubule length = (seminiferous tubule absolute volume [µL])/π(seminiferous tubule radius [µm]^2). Gonadosomatic index (GSI) was calculated as: GSI [%] = testicular mass [g]/bodyweight [g])*100. Statistical significance was *P*<0.05 and a trend defined as *P*<0.10. Non-significant (NS) is used for *P*>0.10.

	Control	Treatment	P-Value
Mean bodyweight (g)	4082.7 ± 180.2	4137.0 ± 210.4	NS
Gross Testis weight (mg)	1476.8 ± 88.7	1820.7 ± 179.2	0.08
Net Testis weight (mg)	1196.2 ± 71.8	1474.8 ± 145.2	0.08
Gonadosomatic index (%)	0.07 ± 0.00	0.09 ± 0.01	NS
Tubule diameter (µm)	227.9 ± 4.4	230.8 ± 5.2	NS
Seminiferous epithelium height (µm)	65.4 ± 1.2	69.7 ± 1.7	0.06
Total tubular length per testis (m)	24.3 ± 2.5	29.1 ± 2.0	NS
Total testis parenchyma volume (µL)	1196.2 ± 71.8	1474.8 ± 145.2	0.08
Testis parenchyma volume density (%)			
Seminiferous tubule	81.4 ± 2.8	83.7 ± 1.4	NS
Tunica propria	2.4 ± 0.2	1.8 ± 0.2	0.06
Seminiferous epithelium	67.6 ± 1.9	71.9 ± 1.0	0.07
Lumen	11.4 ± 2.3	10.0 ± 1.3	NS
Inter-tubular compartment	18.6 ±2.8	16.3 ±1.4	NS
Leydig cells	10.2 ± 2.1	7.0 ± 0.9	NS
Connective tissue	4.8 ± 1.0	6.2 ± 1.3	NS
Blood vessels	1.2 ± 0.3	1.2 ± 0.3	NS
Lymphatic vessels	2.4 ± 0.4	1.9 ± 0.4	NS

The mean (± SEM) seminiferous tubule diameter and epithelium height was 229.5 ± 2.4 µm (range: 208.4 – 265.6 µm) and 67.7 ± 0.9 µm (range: 57.6 – 77.7 µm), respectively. The mean (± SEM) seminiferous tubule length per testis was 26.8 ± 1.2 m (range: 14.4 – 39.6 m). Seminiferous tubule length was similar between the treatment and control groups (Table 3.3). While testis weight and seminiferous tubule length were positively correlated (r^2 = 0.81, *P*<0.001), seminiferous tubule diameter and testis weight were not (r^2 = 0.23, *P*=0.22). Seminiferous tubule diameter, epithelium height, and total length did not differ between the control and treatment groups (Table 3.3), although there was a trend for the seminiferous tubule epithelium height to be higher in the treatment group.

Testis parenchyma comprised mostly of seminiferous tubules (82.6 ± 1.3%, range: 66.2 – 91.9%) and Leydig cells (8.5 ± 0.9%, range: 3.1 – 22.0%), with Leydig cells occupying ~49% of the inter-tubular compartment. Testicular parenchyma volume densities did not differ between the control and treatment groups (Table 3.3). However, the volumes and densities of seminiferous epithelium and tunica propria in the treatment group tended to be higher and lower than the control group, respectively (Table 3.3).

3.2.4 Sertoli and germ cell assessments

The mean (± SEM) number of Sertoli cells per seminiferous tubule cross section was 18.3 ± 0.5 (range: 13.2 – 23.9). Cats in the treatment group had fewer Sertoli cells per seminiferous tubule cross section than those in the control group (P=0.04; Table 3.4). On average, there were approximately 123.3 x 10^6 Sertoli cells per testis and 91.7 x 10^6 Sertoli cells per gram of testis. The number of Sertoli cells per cross-section (P=0.04) and the number of Sertoli cells per gram of testis was lower in the treatment group (P<0.001; Table 3.4).

Table 3.4 Sertoli and germ cell type (spermatogonia, spermatocytes, round spermatids, and elongate spermatids) parameters from the cats in the control (n=7) and cats treated with 1 mg/kg prednisolone for 50 days (n=8) groups. Non-significant (NS) is used for P>0.10.

Cell type	Control	Treatment	P-Value
Sertoli cells			
Per tubule cross section	19.7 ± 0.8	17.1 ± 0.9	0.04
Per testis (x10^6)	119.1 ± 10.9	127.3 ± 8.4	NS
Per gram of testis (x10^6)	98.7 ± 2.9	85.6 ± 4.2	<0.001
Germ cells			
Number of germ cells/tubule cross section	185.3 ± 9.4	196.5 ± 10.8	NS
Number of spermatids/tubule cross section	110.6 ± 7.1	122.9 ± 6.8	NS
Relative germ cell abundance (%)			
Spermatogonia	9.4 ± 0.5	9.5 ± 0.4	NS
Spermatocytes	31.0 ± 1.3	27.9 ± 0.6	0.06
Round Spermatids	31.1 ± 1.5	34.7 ± 1.0	0.08
Elongate Spermatids	28.4 ± 1.4	27.9 ± 0.9	NS
Sertoli cell load			
Number of germ cells/Sertoli cell	9.4 ± 1.2	11.5 ± 0.8	<0.001
Number of spermatids/Sertoli cell	5.6 ± 0.1	7.2 ± 0.1	<0.001

The mean (± SEM) number of germ cells per seminiferous tubule cross section was 191.3 ± 5.1 (range: 140.2 – 251.2), with 117.2 ± 3.7 (range: 76.4 – 160.0) spermatids per tubule cross section. Both the total number of germ cells and spermatids per tubule cross section did not differ between the control and treatment group. The total number of germ cells per tubule cross section was correlated with tubule diameter (r^2 = 0.64, P<0.001), as was the number of spermatids per tubule cross section (r^2 = 0.51, P = 0.004). Round spermatids were the most abundant germ cell (33.0%), followed by spermatocytes (29.4%) and elongate spermatids (28.1%), with spermatogonia being present in much lower proportion (9.5%). The relative abundances of the different germ cell types did not differ significantly between the treatment and control groups (Table 3.4), but there was a tendency for spermatocytes and round spermatids to have a higher relative abundance in the treatment group.

There were an estimated 10.5 ± 1.3 (mean ± SEM, range: 6.6 – 12.1) germ cells (spermatogonia, spermatocytes, and spermatids) per Sertoli cell and 6.4 ± 1.0 (mean ± SEM, range: 3.9 – 7.6) spermatids per Sertoli cell. The number of Sertoli cells per gram of testis was negatively correlated with both the number of germ cells (r^2 = -0.69, P<0.001) and spermatids (r^2= -0.53, P<0.002) per Sertoli cell. Sertoli cell load (i.e., number of total germ cells per Sertoli cell) was higher in the treatment group than the control group (P<0.001; Figure 3.7; Table 3.4). Sertoli cell load was

positively correlated with the mean percentage of morphologically abnormal sperm (rho = 0.67, $P<0.001$), as well as the percentage of morphologically abnormal sperm in the caput (rho = 0.58, $P<0.001$) and cauda (rho = 0.69, $P<0.001$) regions of the epididymis. The number of spermatids per Sertoli cell was also positively correlated with the percentage of abnormal sperm in both the caput (rho = 0.62, $P<0.001$) and cauda (rho = 0.78, $P<0.001$) regions of the epididymis.

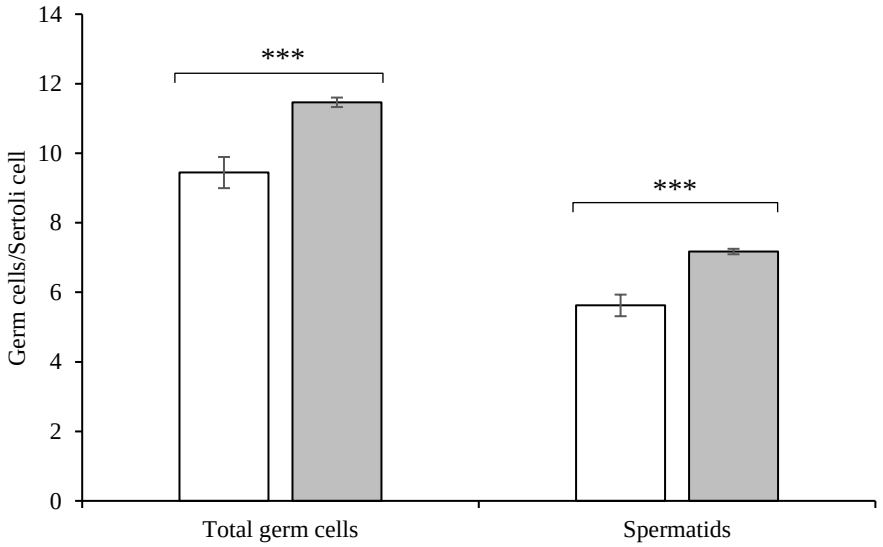

Figure 3.7 The mean (± SEM) number of germ cells (spermatogonia, spermatocytes, round spermatids, and elongate spermatids) and spermatids per Sertoli cell in the untreated control cats (white bars; n=7) and cats treated with 1 mg/kg prednisolone for 50 days (grey bars; n=8). ***P<0.001.

3.2.5 Sertoli and germ cell apoptosis: TUNEL assay

The TUNEL assay stained apoptotic cells in both the cat testis and chicken small intestine (control; Figure 3.8). While there was no staining in the negative controls (rTdT omitted from assay), DNase-treated tissues (positive controls) showed intense staining (Figure 3.8). The total number of apoptotic cells per tubule cross-section was similar between prednisolone-treated (0.97 ± 0.12 apoptotic cells) and control (1.16 ± 0.05 apoptotic cells) cats. However, Sertoli cell apoptosis was higher in the treatment group ($P<0.001$; Figure 3.9). There were fewer apoptotic germ cells per tubule cross-section in the prednisolone-treated cats ($P=0.03$) than control cats, although only the number of apoptotic spermatocytes differed between the two groups of cats ($P=0.04$; Figure 3.9).

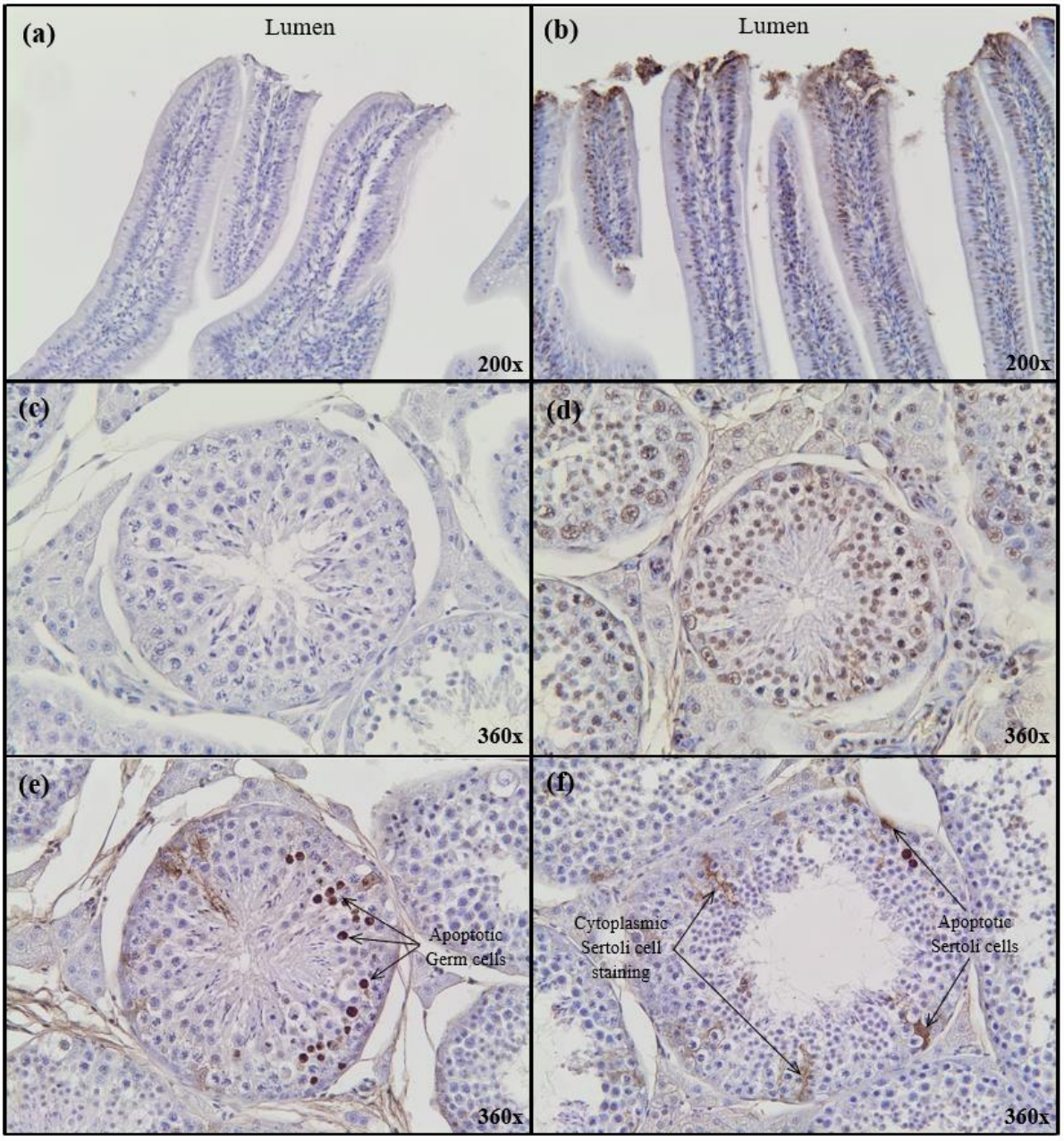

Figure 3.8 TUNEL apoptosis assay of (a) small intestine of chicken with recombinant terminal deoxynucleotidyl transferase (rTdT) omitted (i.e., negative control), (b) DNase-treated small intestine of chicken (i.e., positive control), (c) cat testis with rTdT omitted (i.e., negative control), (d) DNase-treated cat testis (i.e., positive control), (e) seminiferous tubule cross-section with apoptotic germ cells, and (f) seminiferous tubule cross-section with cytoplasmic Sertoli cell staining (non-apoptotic). Apoptotic cells are stained brown.

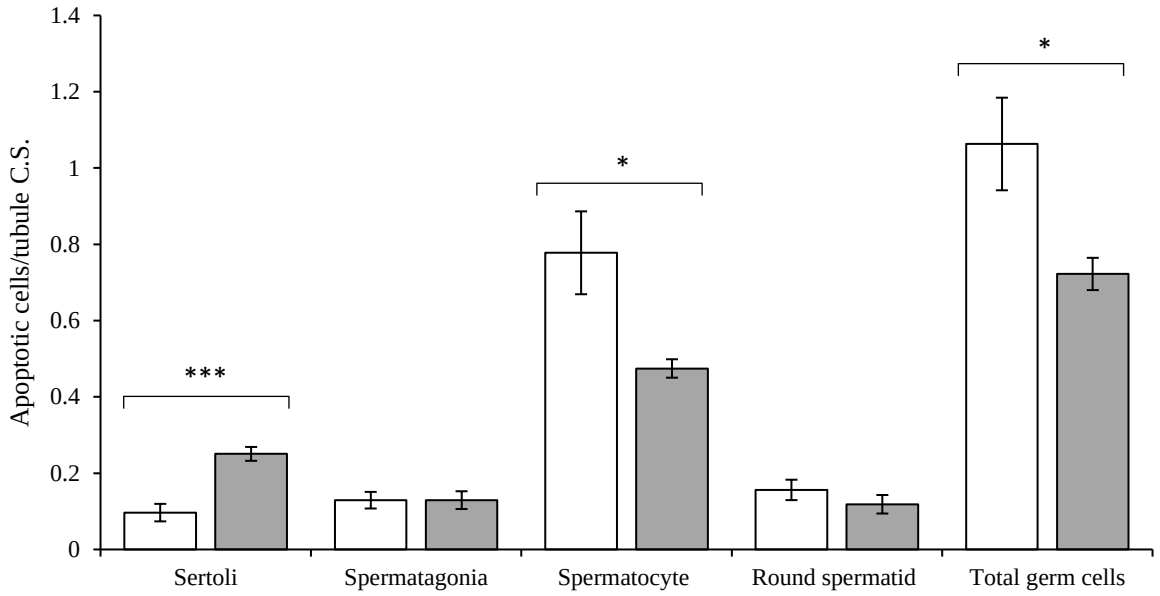

Figure 3.9 Mean (± SEM) number of apoptotic (as identified using a TUNEL assay) Sertoli and germ cells (spermatogonia, spermatocytes, and round spermatids) per seminiferous tubule cross-section (C.S.) in cat testes of untreated control cats (white bars; *n*=7) and cats treated with 1 mg/kg prednisolone for 50 days (grey bars; *n*=8). Note that elongate spermatids were not included, as TUNEL staining was difficult to accurately quantify in these cells. **P*<0.05, ***P*<0.001.

3.4 Discussion

This is the first study to examine the effects of a simulated endocrine stress response (GC treatment) on testicular function in domestic male cats. The hypothesis that elevated GC (as may derive from captivity-related stress) have a detrimental effect on testicular function in domestic cat was supported. While sperm motility was similar between the treatment and control groups, cats given prednisolone exhibited a higher proportion of primary and secondary sperm abnormalities in both the caput and cauda epididymis (Figure 3.6). The prednisolone treatment did not significantly alter testicular histomorphometric parameters and volumetrics (Table 3.3). However, cats in the treatment group had fewer Sertoli cells per seminiferous tubule cross section and a higher Sertoli cell load (i.e., number of germ cells per Sertoli cell). Interestingly, a higher Sertoli cell load was positively correlated with the percentage of morphologically abnormal sperm in the epididymis.

Over the treatment period (i.e., 50 days), glucose concentrations were higher in prednisolone-treated cats than in control cats. This was not surprising given that GC administration is well known to increase blood glucose concentrations via several pathways including promoting gluconeogenesis in the liver, inhibiting glucose uptake by skeletal muscle and adipose tissue, and suppressing insulin secretion pancreatic β cells (Kuo *et al.*, 2015). Prednisolone-treated cats had lower serum cortisol concentrations, which agrees with past literature suggesting the exogenous GC decrease endogenous cortisol concentrations (Middleton *et al.*, 1987). Prednisolone and prednisone were undetectable in the

serum of both prednisolone-treated and control animals. There is conflicting data on the half-life of prednisolone, with veterinary textbooks reporting a 12-36 hour half-life (Behrend and Kemppainen, 1997; Feldman and Nelson, 2004) and human pharmacokinetic study reporting 2-3 hour half-life (Rose *et al.*, 1981). Blood samples were taken 22-24 hours after the prednisolone treatment was administered. If the prednisolone half-life is in fact 2-3 hours, then it is likely that circulating prednisolone concentrations were below the limit of quantification (i.e., <0.25 ng/mL) by the time sampling occurred.

Testosterone concentrations did not differ between control and treatment animals. It was possible that testosterone concentrations changed as prednisolone concentrations declined after administration. For future studies, it would be advisable to collect blood samples within 2-4 hours of GC administration to determine whether the lack of effect on testosterone concentration is real or an artefact of our experimental design. Despite no change in serum testosterone or prednisolone concentrations between prednisolone-treated and control cats, other effects of the treatment were observed on parameters such as sperm abnormalities, Sertoli cell numbers, and Sertoli cell load.

The percentage of morphologically abnormal sperm was significantly higher in the epididymides of cats given prednisolone. In fact, both primary and secondary abnormalities were more prevalent in the treatment cats, thus implying that the GC treatment adversely affected both spermatogenesis and sperm maturation, respectively (Pukazhenthi *et al.*, 2001; Pukazhenthi *et al.*, 2006b; Crosier *et al.*, 2009; Müller *et al.*, 2012). Primary sperm abnormalities, which were significantly increased in the prednisolone-treated cats, are often due to impaired Sertoli cell function and/or high Sertoli cell loads (i.e., too many germ cells per Sertoli cell; Mruk and Cheng, 2004; Pukazhenthi *et al.*, 2006b). Sertoli cell load was positively correlated with the percentage of abnormal sperm in the epididymis of the cats in the present study. Abnormal testosterone concentrations can lead to an increase in primary sperm abnormalities, as testosterone is required for germ cell-Sertoli cell adhesion (Mruk and Cheng, 2004; Wang *et al.*, 2006). For example, AR-knockout mice only exhibit one of the eight proteins involved in the desmosome junction between germ cells and Sertoli cells (Wang *et al.*, 2006). Incomplete desmosome junctions have been linked to impaired Sertoli cell function, incomplete sperm development, and thus a high proportion of morphologically abnormal sperm (Mruk and Cheng, 2004; Wang *et al.*, 2006). Secondary abnormalities are typically a consequence of abnormal developmental conditions during sperm passage through the epididymis (e.g., hormone concentrations, seminal plasma composition and pH; Pukazhenthi *et al.*, 2001; Pukazhenthi *et al.*, 2006b). Testosterone slows the transport of sperm through the epididymis, so it is not surprising that abnormally low testosterone concentrations have been linked to an increase in the proportion of secondary abnormalities such as proximal cytoplasmic droplets of mice (Meistrich *et al.*, 1975). While cats given prednisolone exhibited a higher percentage of primary and secondary sperm abnormalities in this study, testosterone concentrations were similar between the control and treatment

cats. The increased proportions of morphologically abnormal sperm may adversely affect the fertility of the cats, as abnormal sperm are rarely involved in the fertilisation process and can even disrupt the function of structurally normal sperm (Howard *et al.*, 1990; Long *et al.*, 1996; Pukazhenthi *et al.*, 2006b).

Atypically low testosterone concentrations have been linked to an increased prevalence of sperm abnormalities (Meistrich *et al.*, 1975; Müller *et al.*, 2012). As discussed above for prednisolone, it is possible that differences in the testosterone concentrations of control and treatment animals were missed due to sampling interval. It is also conceivable that GC did not affect testosterone concentration, but altered the concentrations of testicular androgen receptors and/or androgen-binding protein (ABP) instead. Androgen binding protein has an important role of regulating testicular androgen concentrations and concentrating testosterone in the testis and the epididymis (Munell *et al.*, 2002). Interestingly, abnormally low testicular ABP concentrations have been linked to impaired or overwhelmed (too many germ cells) Sertoli cells (Mruk and Cheng, 2004; Johnson *et al.*, 2008).

Sertoli cells perform a wide variety of functions including the formation and regulation of the blood-testis barrier, germ cells support (nutritional, hormonal, and structural), production of ABP, regulation of the translocation of germ cells across the seminiferous epithelium, spermiation, and phagocytosis of apoptotic germ cells (Mruk and Cheng, 2004; Johnson *et al.*, 2008). Overexerted Sertoli cells (i.e., those with too many germ cells) cannot perform these functions as efficiently and thus germ cell development is impaired (Mruk and Cheng, 2004; Johnson *et al.*, 2008). In the current study, both the mean number of germ cells (9.4 ± 1.2) and the mean number of spermatids (5.6 ± 0.1) per Sertoli cell in control animals were comparable to past studies: 9.8 ± 0.8 total germ cells and 5.1 ± 0.6 spermatids per Sertoli cell (França and Godinho, 2003), and 5.2 ± 0.3 spermatids per Sertoli cell (Müller *et al.*, 2012). However, cats treated with prednisolone had a higher Sertoli cell load which may in part explain the sperm morphological abnormalities detected in these animals, independently of testosterone concentrations.

The increase in Sertoli cell load could be caused by one of two things: either an increased number of germ cells or a reduction in Sertoli cell number. The total number of germ cells per seminiferous tubule cross-section, as well as the relative abundance of the different germ cells (spermatogonia, spermatocytes, round spermatids, and elongate spermatids), were similar between treatment and control cats. Interestingly, germ cell apoptosis was lower in prednisolone-treated cats, specifically the number of apoptotic spermatocytes per tubule cross-section. Jewgenow *et al.* (2009) reported reduced spermatocyte apoptosis in teratospermic domestic cats, with the authors suggesting that the high incidence of abnormal sperm observed in the ejaculates of these cats may be due, in part, to the reduced elimination of morphologically abnormal sperm via apoptosis. Reduced germ cell apoptosis

may also explain the higher proportion of abnormal sperm in the epididymides of prednisolone-treated cats.

Regarding reductions in the number of Sertoli cells as a leading cause of increase cell load, the present study showed that cats given prednisolone exhibited fewer Sertoli cells per tubule cross-section and per gram of testicular tissue. This may be at least partly explained by the higher number of apoptotic Sertoli cells per tubule cross-section present in the testis of prednisolone-treated cats. The difference in the number of Sertoli cells per tube cross-section of control and treatment cats was surprising, given that Sertoli cell numbers are considered to be stable in adult mammals (Johnson *et al.*, 2008). It is typically thought that Sertoli cells do not proliferate in adult mammals, although there is some evidence to indicate that Sertoli cell proliferation may occur under certain circumstances (Johnson *et al.*, 2008). Taken altogether, the potential GC-induced reduction in Sertoli cell numbers would be particularly concerning as it suggests that prolonged periods of elevated GC concentrations could have a permanent or prolonged effect on testicular function.

It is worth noting that caution is needed when considering the estimated total number of Sertoli cell per testis or gram of testis, since these estimates are likely to be imprecise as it is difficult to accurately correct for the inconsistent size and/or shape of Sertoli cells. Indeed, the total number of Sertoli cells per testis or gram of testis has varied considerably across different publications studies: $31.6 \pm 6.4 \times 10^6$ Sertoli cells per g of testis (França and Godinho, 2003), $54.7 \pm 5.5 \times 10^6$ Sertoli cells per g of testis (Müller *et al.*, 2012), and $92.2 \pm 2.8 \times 10^6$ Sertoli cells per g of testis (present study). Thus, it would be recommended to focus primarily on a more accurate or relative measurement of Sertoli cell number, such as the number of Sertoli cells per tubule cross section

3.5 Conclusions

Prednisolone treatment adversely affected sperm quality in cats independently of testosterone concentration, with treatment cats exhibiting a higher proportion of morphologically abnormal sperm in the epididymides. Both primary and secondary sperm abnormalities were higher in the prednisolone-treated cats, suggesting that spermatogenesis and sperm maturation were affected by the treatment. The prednisolone treatment did not appear to alter total number and relative abundance of spermatogonia, spermatocytes, or spermatids. However, cats given prednisolone had fewer Sertoli cells per tubule cross-section and a higher Sertoli cell load (number of germ cells per Sertoli cell), which could have contributed towards to increased proportion of morphologically abnormal sperm in the prednisolone-treated cats. Testicular tissue was retained to examine any effects of the prednisolone treatment on gonadotrophin receptor and ABP expression, as this may provide further insight into pathways by which GC adversely affect testicular functions.

Chapter 4

The effects of a simulated endocrine stress response on the ovarian function of domestic cats (*Felis catus*) undergoing exogenous gonadotrophin treatments

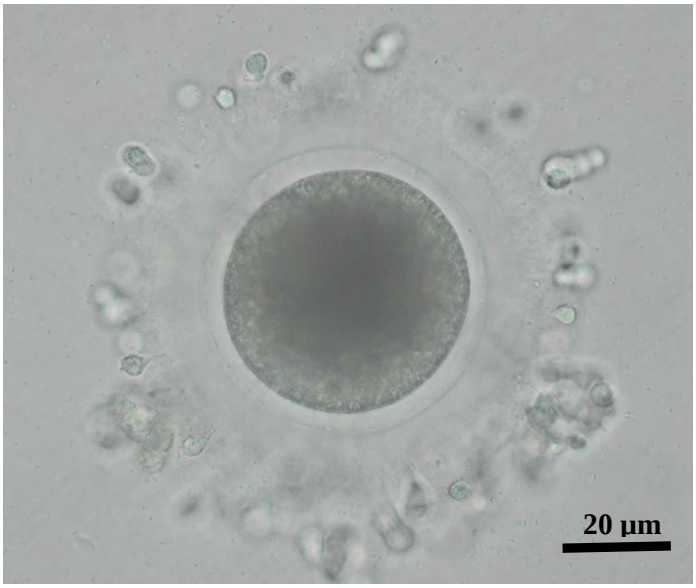

Photo of feline oocyte (400 X magnification), by Chris Andrews.

Chapter 4: The effects of a simulated endocrine stress response on the ovarian function of cats (*Felis catus*) undergoing exogenous gonadotrophin treatments

4.0 Abstract

This study examined the effects of a simulated endocrine stress response (i.e., glucocorticoid (GC) treatments) on the ovarian function and oocyte quality of domestic cats undergoing ovarian stimulation treatment. It was hypothesised that the number of ovulations following exogenous gonadotrophin treatments and/or oocyte quality will be impaired in cats given a GC treatment. Entire female cats were divided into treatment (n=6) and control (n=6) groups. Cats in the treatment group were given 1 mg/kg/day prednisolone orally for 45 days. All cats were given 0.088 mg/kg/day progesterone orally from Day 0 (first day of prednisolone treatment) to Day 37 of the study to suppress follicular growth. On Day 40, the cats were intramuscularly treated with 75 IU equine chorionic gonadotrophin (eCG) to induce follicular growth, followed by 50 IU human chorionic gonadotrophin (hCG) 80 hours later to induce ovulation. Cats were ovariohysterectomised 30 hours after the hCG treatment and the uteri and ovaries collected. The ovarian responses were graded from 1-4 (1 (excellent) = multiple corpora haemorrhagica (CH)/fresh corpora lutea (CL), 2 (good) = multiple CH/fresh CL with large (≥2 mm) follicles, 3 (fair) = mix of CH/fresh CL and aged CL, or 4 (poor) = no ovulatory response). Ovaries were fixed in 4% paraformaldehyde and embedded in paraffin. Oocytes were recovered by retrograde flushing of the uterine tubes using 37°C phosphate buffered saline with 5% foetal bovine serum. Oocyte diameter and zona pellucida (ZP) thickness were measured using light microscopy. Each oocyte was given a total oocyte score (TOS: 0-8, 8=best) based on four parameters: oocyte morphology, size, ooplasm uniformity and granularity, and ZP thickness and thickness variation. Blood samples were collected on Days 0, 10, 30, and 40, prior to the hCG treatment, and at the time of ovariohysterectomy. Serum oestradiol, progesterone, cortisol, glucose, and prednisolone concentrations were assessed. The induction of follicular growth and ovulation was confirmed in all cats by oestradiol concentrations and morphological assessment of the ovaries, with a mean of 10.5 ± 1.1 ovulations per cat. Body weight, ovarian weight and volume, graded ovarian response, number of ovulations, and oocyte recovery did not differ between the two groups of cats. Oocyte diameter was comparable between the two groups, but the ZP was thinner in the treatment group (3.1 ± 0.3 μm vs. 4.1 ± 0.3 μm, P=0.03). While the TOS was similar between treatment and control cats, the ooplasm grade was lower in the treatment group (1.5 ± 0.1 vs. 1.9 ± 0.1, P=0.01). There was also a tendency for ZP grade to be poorer in the treatment group (0.8 ± 0.1 vs. 1.2 ± 0.2; P=0.08). However, it is unclear whether these morphological changes would affect fertility. In the future, *in vitro* fertilisation rates and embryo formation need to be assessed to confirm that GC treatment impairs fertility in female cats.

4.1 Introduction

Captive breeding programs are a vital component in the conservation strategies for felids, but these programs are often hindered by poor reproductive performance (Mellen, 1991; Terio *et al.*, 2004; Brown, 2006; Moreira *et al.*, 2007; Fanson *et al.*, 2010; Brown, 2011). Natural breeding is often limited due to mate incompatibility and the logistic challenges of moving breeding animals between captive breeding institutes (Howard and Wildt, 2009). Assisted reproductive technologies (ART) offer a means of improving the efficiency of captive breeding programs and transferring genetic material between *in situ* felid populations, and perhaps between *in situ* and *ex situ* populations (Howard and Wildt, 2009). Unfortunately, ART have generally had poor success rates in felids, even when ovarian function is controlled using exogenous progestins and gonadotrophins (Swanson *et al.*, 1997; Pelican *et al.*, 2006; Pelican *et al.*, 2008; Howard and Wildt, 2009; Pelican *et al.*, 2010; Stewart *et al.*, 2012).

A major factor hindering the success of both natural breeding and ART in felids is the effect of captivity-related stress on the reproductive function of female felids. Captive felids exhibit basal glucocorticoid (GC) concentrations that are more than two times higher than their wild conspecifics, which indicates that the captive environment is stressful (Wielebnowski *et al.*, 2002; Terio *et al.*, 2004; Fanson *et al.*, 2012). Public exhibition has been found to increase the basal GC concentrations of cheetahs (*Acinonyx jubatus*) and clouded leopards (*Neofelis nebulosa*; Wielebnowski *et al.*, 2002b; Koester *et al.*, 2015).

The high levels of GC observed in captive felids have been linked to ovarian quiescence and decreased oestradiol production (Jurke *et al.*, 1997; Moreira *et al.*, 2007). It has been shown that moving acyclic females (with high basal GC concentrations) into more enriched enclosures, or taking them off public exhibition, decreased faecal GC concentrations and, more importantly, lead to the resumption of ovarian cyclicity (Jurke *et al.*, 1997; Moreira *et al.*, 2007). It is likely that the high basal GC observed in captive felids would also adversely affect follicular growth and oocyte quality (i.e., fertility). In other mammalian species (e.g., rats (*Rattus norvegicus*) and mice (*Mus musculus*)), GC have been shown to act on all levels of the hypothalamic-pituitary-gonadal axis to supress ovarian steroidogenesis and gametogenesis, disrupt ovulation, and delay the onset of meiotic maturation of oocytes in response to the pre-ovulatory luteinising hormone surge (Hsueh and Erickson, 1978; Kawate *et al.*, 1993; Tetsuka *et al.*, 1999; Yang *et al.*, 1999; Gore *et al.*, 2006; Van Merris *et al.*, 2007; Kirby *et al.*, 2009; Whirledge and Cidlowski, 2010). While there would be clear value in understanding these processes, no research has yet been conducted on the physiological effects of high basal GC concentrations on fertility of female felids.

This study aims to determine the effects of a simulated endocrine stress response (i.e., exogenous GC treatment) on the ovarian function and oocyte quality of female domestic cats undergoing a typical

exogenous gonadotrophin treatment regime for ovarian stimulation. The focus of this study was to use the domestic cat as a model for examining the effects of elevated GC on ovarian function and fertility of female non-domestic felids. It is hypothesised that the number of ovulations following exogenous gonadotrophin treatments and/or oocyte quality will be reduced in cats given a GC treatment.

4.2 Materials and methods

4.2.1 Animals

Twelve healthy, intact female domestic cats that aged 2.50 ± 0.22 (1.54 – 3.96) years and weighed (3.12 ± 0.12 kg (range: 2.57 – 3.87 kg) were used for this trial. The cats were housed in mixed-sex groups of eight in a purpose-built colony cage at the Centre for Feline Nutrition, Massey University, Palmerston North, New Zealand (175◦3'E, lat. 40°22'S, long.). The cats were fed a complete and balanced (AAFCO, 2020) commercial moist (canned) feline diet (Kraft Heinz Wattie's Ltd., Hastings, New Zealand) and had *ad libitum* access to water. The husbandry of the cats complied with the Animal Welfare (Cats) Code of Welfare (Anonymous, 2007) and all research was conducted in accordance with Massey University Animal Ethics Committee protocol number 19/10.

4.2.2 Experimental design

The cats were allocated randomly (using www.randomizer.org) into either the treatment group (*n*=6) and control group (*n*=6). Cats in the treatment group were given 1 mg/kg oral prednisolone (Redipred: Aspen Pharmacare Australia Pty. Ltd., Saint Leonards, NSW, Australia) daily for 45 days. The duration of prednisolone treatment was determined by the hormonal regime used to control ovarian functions (Stewart *et al.*, 2012). The effective cortisol replacement dose using prednisolone is estimated to be 0.44 mg/kg/day in cats (França and Godinho, 2003), thus 1 mg/kg/day prednisolone was selected as it would be comparable to the elevated cortisol concentrations associated captivity-related stress in endangered felids (2 – 2.5 times above basal concentrations; Jurke *et al.*, 1997; Wielebnowski *et al.*, 2002a; Moreira *et al.*, 2007).

All cats (*n*=12) were exposed to an ovarian stimulation regime previously described (Stewart *et al.*, 2012), with slight modifications to the gonadotrophin doses administered. Cats were treated orally with 0.088 mg/kg/day altrenogest (Altreno Oral: Caledonia Holdings Ltd, Auckland, New Zealand), an oral progestin, from Day 0 to 36 of the trial to suppress ovarian activity. Altrenogest treatment was stopped three days prior to exogenous gonadotrophin treatments. On Day 40, the cats were given an intramuscular (i.m.) injection of 75 IU of equine chorionic gonadotrophin (eCG; Novormon: Syntex, Palo Alto, CA, USA) to promote follicular growth, followed by 50 IU human chorionic gonadotrophin (hCG; Chorulon: Merck Animal Health, Madison, NJ, USA) i.m. 80 hours later, to induce ovulation.

The cats were ovariohysterectomised 30-31 hours after the hCG treatment. The cats were pre-medicated with a single i.m. injection of 0.05 mg/kg dexmedetomidine (Dexdomitor: Zoetis Ltd., Auckland, New Zealand), 3.0 mg/kg ketamine (Phoenix Pharm Distributors Ltd., Auckland, New Zealand) and 0.3 mg/kg morphine (DBL™, Pfizer Inc., New York, NY, USA). Anaesthesia was induced using 4.0 mg/kg propofol and maintained using isoflurane (Bayer New Zealand Ltd., Auckland, New Zealand). Once anaesthetised, the cats were ovariohysterectomised and the uteri and ovaries collected. Following ovariohysterectomy, the cats were given an i.m. injection of 0.025 mg/kg atipamezole (Atipam: Jurox Pty Ltd., Rutherford, NSW, Australia; medetomidine reversal agent). All cats were given a subcutaneous injection of 0.3 mg/kg morphine for post-operative analgesia. Lastly, cats in the control group were given 0.5 mg/kg/day meloxicam orally (Metacam: Boehringer Ingelheim, Auckland, New Zealand) for three days after ovariohysterectomy. It is unsafe to administer non-steroidal anti-inflammatory drugs to cats treated with prednisolone, thus cats in the treatment group were given 0.03 mg/kg buprenorphine (Temgesic; Indivior Pty. Ltd., Macquarie Park, Australia) sublingually twice a day for two days following ovariohysterectomy. The cats in the treatment group were gradually tapered off the oral prednisolone, initially receiving a reduced dose of 0.5 mg/kg daily for six days following castration (Day 45-51), then 0.5 mg/kg every second day for a further six days (Days 52-58).

4.2.3 Ovarian collection and histological assessment

The collected ovaries were separated from the adjacent tissue and the response of each ovary to the exogenous gonadotrophin regime graded according to Stewart *et al.* (2012). Grade 1 (excellent) was characterised by ovarian responses which exhibited multiple corpora haemorrhagica (CH)/fresh corpora lutea (CL) and no large follicles (>2 mm). Grade 2 (good) responses that had a mix of CH/fresh CL and large follicles. Grade 3 (fair) responses had a mixed cohort of aged and fresh CL with or without large follicles. Grade 4 (poor) responses that did not show signs of ovulation. Each ovary was weighed, had its dimensions measured, and volume calculated (volume [mm^3] = length [mm]*width $[mm]^2$*0.524). Gross ovary weights were combined and the gonadosomatic index (GSI) was calculated as: GSI (%) = total ovarian mass [g]/bodyweight [g])*100. Ovarian tissue was then fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 48 hours and subsequently embedded in paraffin wax.

4.2.4 Oocyte collection and fixation

Each oviduct was flushed within 5 minutes of retrieval with 37ºC PBS (720 mOsm and pH 7.2) containing 5% foetal bovine serum (Life Sciences NZ Ltd, Auckland, New Zealand) and the oocytes were collected in a petri dish. Oocytes were examined at 400 X magnification and imaged using an Olympus BX 51 microscope, Olympus SC100 camera, and the Olympus CellSens imaging software (Olympus Corporation, Tokyo, Japan). The oocyte recovery rate was determined for each cat as the number of oocytes retrieved devided by the total number of CH/CL on the ovaries. The oocyte

diameter (average of two perpendicular measurements) and zona pellucida (ZP) thickness (average of four measurements) was recorded (Figure 4.1). The ZP thickness variation (ZPTV) was also calculated for each oocyte: ZPTV = (ZP max thickness [μm] – ZP mean thickness [μm])/ZP mean thickness [μm]*100%.

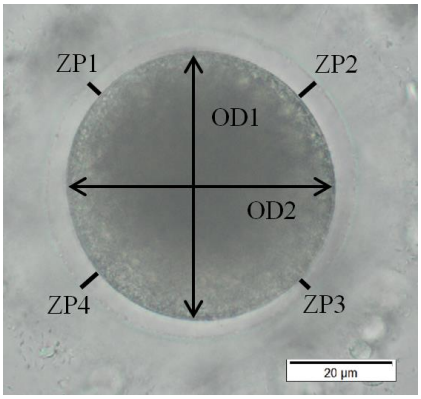

Figure 4.1 Measurements of oocyte diameter (OD1 and OD2) and zona pellucida thickness (ZP1, ZP2, ZP3, and ZP4) in a cat oocyte, which were averaged to provide a single measure of oocyte diameter and zona pellucida thickness.

The oocytes were graded using a modified version of a method described previously for humans (Lazzaroni-Tealdi *et al.*, 2015). Oocytes were evaluated using four parameters: oocyte morphology, oocyte size, ooplasm characteristics, and ZP morphology (Table 4.1). Each parameter was graded as poor (0), moderate (1), or good (2; Table 4.1). The grades for each of the parameter were then summed to provide the total oocyte score (TOS), which ranged from 0 (poor) – 8 (good)). Good quality oocytes were defined by a TOS of 7-8. Moderate quality oocytes had a TOS of 4-6, while poor quality oocytes had a TOS of 0-3. Figure 4.2 provides examples of good, moderate, and poor quality oocytes.

Table 4.1 Parameters and criteria for grading cat oocytes. Note that the definitions of each grade for oocyte size and zona pellucida (ZP) characteristics were derived from the mean (± Stdev) of the oocyte diameter and ZP thickness for the oocytes collected in the present study.

Parameter	Grade		
	0 (poor)	1 (moderate)	2 (good)
Oocyte morphology	Very ovoid or completely misshapen	Slightly ovoid	Round
Oocyte size (diameter)	<45 μm OR >65 μm	45<50 μm OR 60>65 μm	50<60 μm
Ooplasm characteristics	Very granular or vacuolated or several inclusions.	Slightly granular or vacuolated or few inclusions	Absence of granularity or inclusions
ZP characteristics	1) If very thin <1.5 μm or thick >6.0 μm 2) Very -inconsistent shape around oocyte (stdev) across four ZP measurements of >2.0 μm). 3) abnormal appearance.	1) Slightly thin (1.5<2.0 μm) or thick (5.0<6.0 μm) 2) Slightly inconsistent shape around oocyte (Stdev across four ZP measurements of 1.0<2.0 μm 3) Slightly abnormal appearance	1) Normal thickness (2.0<5.0 μm) 2) Consistent shape around oocyte (Stdev across four ZP measurements <1.0 μm). 3) Normal appearance.

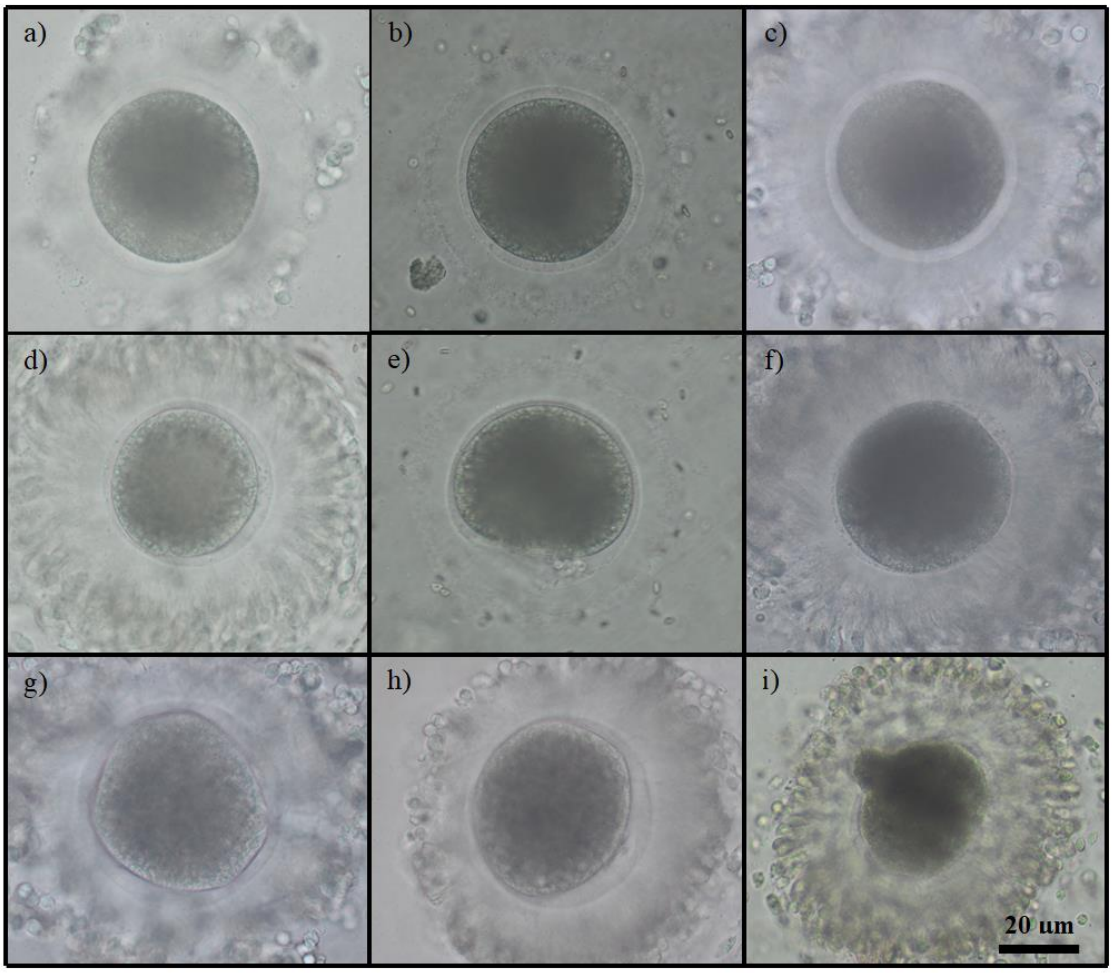

Figure 4.2 Oocytes collected from cats following the exogenous stimulation of follicular growth and ovulation (400 X magnification). Oocytes were graded on four parameters (morphology, size, ooplasm, and ZP), with each parameter being graded from 0 – 2 (2=best). The total oocyte score (TOS) was the sum of the grades for the four oocyte parameters. (a,b,c) Good quality oocytes (TOS of 7 or 8), (d,e,f) moderate quality oocytes (TOS of 4 - 6), and (g,h,i) poor quality oocytes (TOS 0-3).

4.2.7 Endocrine assessment

Jugular venepuncture was used to collect 2 mL blood samples from all cats on Days 0 (immediately before first GC treatment), 10, 30 and 40 (immediately before the eCG treatment), and then 80 hours after eCG treatment, and while the cats were under anaesthesia for ovariohysterectomy (Day 45 of the trial).). Local anaesthetic (2% xylocaine gel, Aspen Pharmacare Australia Pty Ltd., Saint Leonards, NSW, Australia) was applied to the neck of the cats prior to blood collection. Blood samples were collected into vacuum tubes (Becton and Dickinson Co., Franklin Lakes, NJ, USA), left for 2-3 hours at room temperature then centrifuged at 2000 rpm for 15 minutes and serum extracted. Serum samples were stored at -80ºC until assessment. Day 30 was considered as baseline for all endocrine assessments as this allowed sufficient time (Day 0-30) for complete ovarian suppression (i.e., no follicular or luteal activity

4.2.7.1 Cortisol

Serum cortisol concentrations were analysed commercially by IDEXX Laboratories (Palmerston North, New Zealand). Cortisol concentrations were analysed using the Immulite® 1000 cortisol immunoassay. Cross-reactivity of the assay was reported as 100% for cortisol, 49% for prednisolone, 21% for methyl-prednisolone, 8.6% for corticosterone, 5.9% prednisone, and <1.0% for all other tested steroids (*n*=21). The analytical range was 2.0 – 500.0 ng/mL and all samples and standards were run in duplicate. The mean intra-assay and inter-assay coefficients of variance (CV) were 7.13 ± 0.45% and 7.88 ± 0.51%, respectively.

4.2.7.2 Glucose

Glucose concentrations were used as an indicator for the efficacy of the prednisolone treatment and were analysed commercially by IDEXX Laboratories (Palmerston North, New Zealand). Serum glucose concentrations were determined using an enzymatic ultraviolet test (Beckman Coulter inc., Brea, CA, USA) and an AU680 clinical chemistry analyser (Beckman Coulter inc., Brea, CA, USA). The analytical range was 10.0 – 800.0 mg/dL. The mean intra-assay and inter-assay CV were 0.58 ± 0.06% and 1.11 ± 0.08%, respectively.

3.2.7.3 Prednisolone

Prednisolone, prednisone, cortisone, and cortisol were measured in the serum samples collected on Days 0, 10, 30, and 45 using ultra-high performance liquid chromatography (UHPLC) with mass spectrometry. The internal standard was cortisol-d4 (60 ng/mL), with 100 µL of the internal standard (in water) being added to 200 µL of blank plasma, standard (standards were 0.25, 0.63, 2.5, 7.5, 25.0, 75.0, 250.0, and 500.0 ng/mL for each steroid), quality control (QC), and collected serum samples. For complete methodology, see Chapter 3.

4.2.7.4 Oestradiol

Serum 17β-oestradiol concentrations were measured using the Cobas® e601 analyser (Hitachi Ltd., Tokyo, Japan) and a commercially available electrochemiluminescence immunoassay (ECLIA), Cobas® Estradiol III (Roche Diagnostics New Zealand, Auckland, New Zealand). Cross-reactivity was reported to be <1.0% for all tested steroids (*n*=35) except for 6-α-hydroxy-oestradiol, which had a cross reactivity of 74.1%. The detection range was 5.0 – 3000.0 pg/mL. Values below the detection threshold were considered to be 0 pg/mL. The mean intra-assay coefficient of variance (CV) for quality controls was 3.2%.

4.2.7.5 Progesterone

Serum progesterone concentrations were measured using the Cobas® e601 analyser (Hitachi Ltd., Tokyo, Japan) and a commercially available ECLIA, Cobas® Progesterone III (Roche Diagnostics New Zealand, Auckland, New Zealand). Cross-reactivity was reported to be <1.0% for all tested steroids (*n*=27) except for 11-deoxycorticosterone, which had a cross reactivity of 3.9%. The

detection range was 0.05 – 60.0 ng/mL. Values below the detection threshold were considered to be 0.0 ng/mL. The mean intra-assay CV for the quality controls was 2.7%.

4.2.6 Statistical analysis

RStudio version 1.0.143 (R Foundation for Statistical Computing, Vienna, Austria) and a significance level of $P<0.05$ was used for all statistical assessments. A trend or tendency was defined as $P<0.1$. Shapiro-Wilk normality tests were used to assess the normality of all data. Due to large standard deviations, Chauvenet's criterion was used to remove outliers from the endocrine data. The effect of treatment with respect to Day of trial on cortisol, glucose, oestradiol, and progesterone concentrations was examined using linear mixed models, but cortisol, oestradiol, and progesterone data were exponentially transformed using the function 'transformTukey' to meet the test requirements (i.e., normality). The bodyweight, ovarian weight, ovarian volume, ovarian response (grade), ovulation rate, oocyte recovery, and oocyte parameters of the control and treatment cats were compared using either a Welch's T-test or Mann-Whitney-Wilcox test depending on normality. The percentage of oocytes in each TOS score of control and treatment cats was compared using a Chi-squared test. All data are presented as mean ± SEM unless stated otherwise. For correlation analyses, Pearson or Spearman correlation coefficients were used depending on the normality of the data.

4.3 Results

The mean bodyweight of the cats was 3.12 ± 0.12 kg (range: 2.57 – 3.87 kg), and bodyweight did not differ between the control (3.12 ± 0.17 kg) and treatment (3.09 ± 0.19 kg) groups. The bodyweights of the cats did not change significantly over the trial, with a mean change in bodyweight of 0.20 ± 0.05 kg (range: -0.01 – 0.43 kg). No adverse side effects of the prednisolone treatment were observed in the treatment cats. Ovarian weight and volume were similar between the control and treatment groups, with a mean ovarian weight and volume of 0.3 ± 0.02 g (range: 0.2 – 0.4 g) and 40.0 ± 2.0 mm^3 (range: 28.6 – 53.7 mm^3), respectively. The GSI did not differ significantly between the treatment and control cats, mean GSI of 0.022 ± 0.002%. Bodyweight was not correlated with either ovarian weight or volume, but there was a strong positive correlation between ovarian weight and volume ($r^2=0.87$, $P<0.001$).

4.3.1 Endocrinology

The mean serum cortisol concentration throughout the study for both treatment groups combined was 17.8 ± 1.7 ng/mL. Baseline (Day 0) cortisol concentrations were similar between the control and treatment cats, with a mean Day 0 concentration of 19.3 ± 4.6 ng/mL. The mean cortisol concentrations from Day 10-45 were lower in the treatment group than in the control group (13.7 ± 2.7 ng/mL and 21.1 ± 2.5 ng/mL, respectively; $P=0.02$). However, there was neither a time nor treatment by time interaction on cortisol concentrations (Figure 4.3). Prednisolone and prednisone were undetectable in all serum samples.

Mean blood glucose concentrations were higher in the treatment group than in the control group (79.1 ± 1.4 mg/dL vs. 73.9 ± 1.6 mg/dL respectively; P=0.02), although glucose concentrations only differed significantly between the groups on Day 44 (Figure 4.3). However, there was no treatment by time interaction for glucose concentrations (P=0.31).

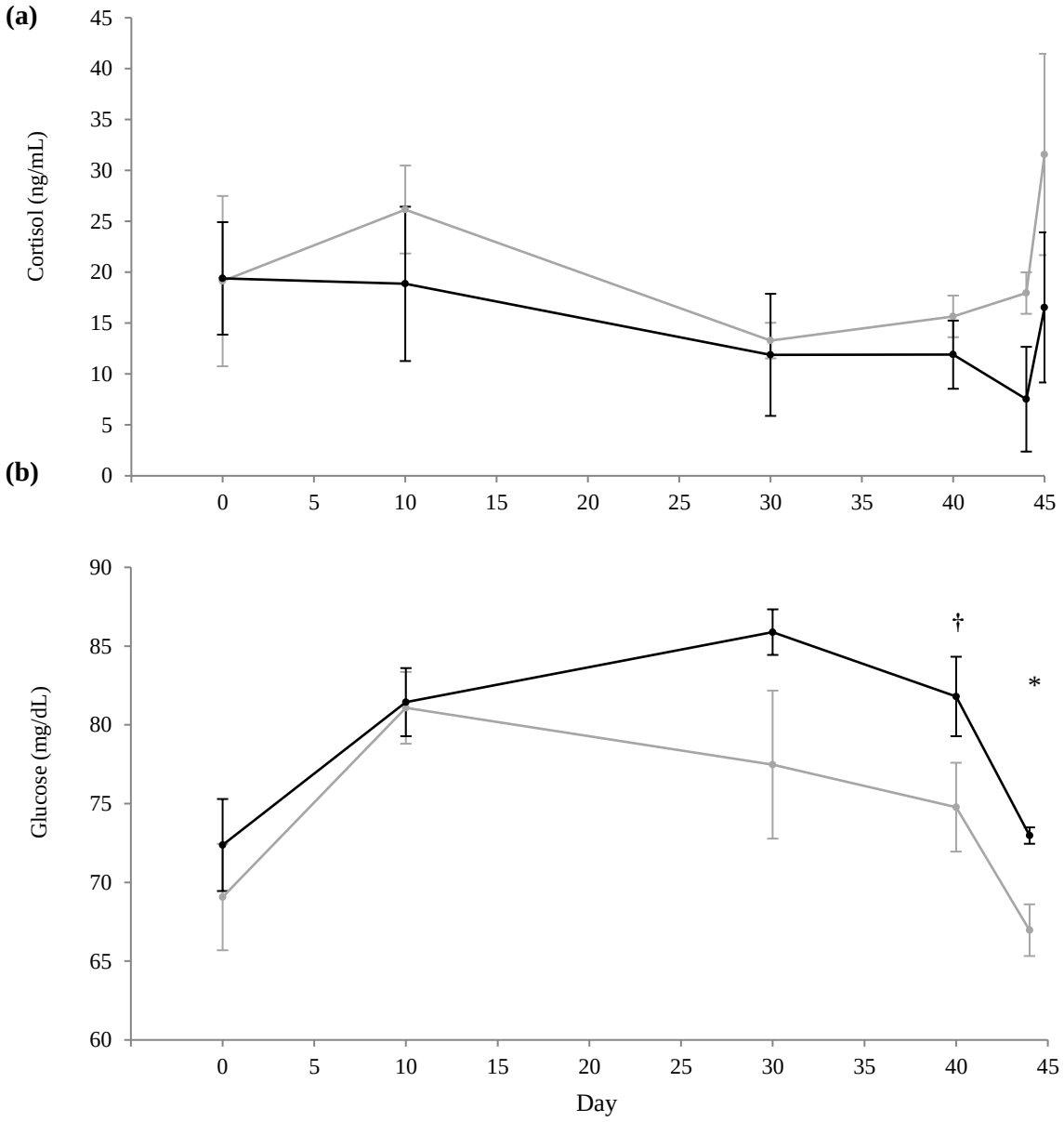

Figure 4.3 Mean (± SEM) serum cortisol (a) and blood glucose (b) concentrations of untreated control cats (grey line; n=6) and the treatment cats (black lines; n=6), which were given 1 mg/kg prednisolone from Day 0-45. Cats were treated with 0.088 mg/kg/day oral progesterone from Day 0-37. On Day 40, the cats were given 75 IU equine chorionic gonadotrophin (eCG) to induced follicular growth, followed by 50 IU human chorionic gonadotrophin (hCG) to induce ovulation 80 hours later. The cats were ovariohysterectomised 24 hours after the hCG treatment (i.e., Day 45). Note that glucose was not analysed on Day 45. Linear mixed models indicated that there was no treatment by time interaction for either cortisol or glucose concentrations, thus treatment and control groups were compared for each day. †P<0.10, *P<0.05.

Serum oestradiol concentrations did not differ between the treatment and control groups, even when time effect was accounted for (Figure 4.4); thus, values from each treatment group were combined for further analysis. The mean oestradiol concentration on Day 0 was 17.8 ± 3.4 pg/ml, which has previously been correlated to a lack of active ovarian follicles (Shille *et al.*, 1979). After 30 days of follicular suppression by the altrenogest treatment, the mean baseline oestradiol concentration (Day 30) was 40.7 ± 5.1 pg/mL. Oestradiol concentrations changed over time ($P<0.001$), but post-hoc analysis indicated that only Day 44 values differed significantly from the other time points. Mean oestradiol concentration on Day 44 was 85.6 ± 14.3 pg/mL, which was 2.3-fold higher than mean baseline (Day 30) concentrations. Blood sample collection of Day 44 of the trial was unable to be collected from one cat; thus, it was not possible to document a peak in serum oestradiol concentrations following the eCG treatment in that female.

Three of the cats in the treatment group had elevated progesterone concentrations (35.3 ± 6.3 ng/ml) on Day 0, suggesting the presence of functional CL (i.e., dioestrus); two of these cats still had elevated progesterone concentrations (12.6 ng/ml and 36.7 ng/ml) on Day 10. Baseline (Day 30) progesterone concentrations were similar between the treatment and control groups (0.38 ± 0.12 ng/mL vs. 0.64 ± 0.09 ng/mL, respectively; $P=0.61$). Progesterone concentrations did not vary significantly over time in either the treatment or control groups. Mean progesterone concentrations (Day 30-45) were lower in the prednisolone-treated cats than control cats (0.57 ± 0.13 ng/mL vs. 1.01 ± 0.12 ng/mL, respectively; $P=0.004$; Figure 4.4). However, progesterone concentration were only significantly lower in the treatment cats than control cats on Day 44 ($P=0.02$), although progesterone concentrations also tended to be lower in the treatment group on Day 40 ($P=0.09$; Figure 4.4).

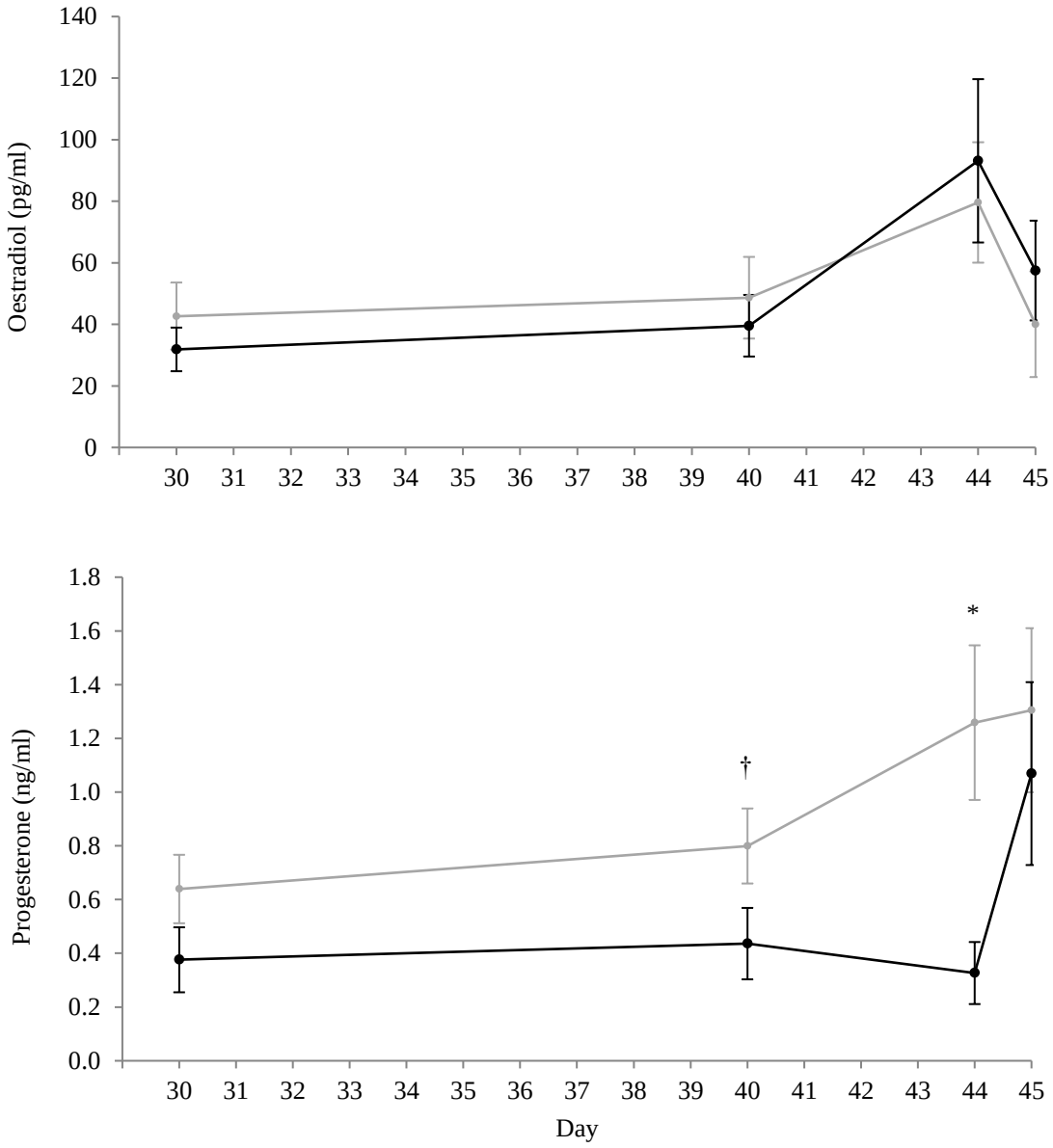

Figure 4.4 Mean (± SEM) serum oestradiol (a) and progesterone (b) concentrations of untreated control cats (grey line; *n*=6) and treatment cats (black lines; *n*=6), which were given 1 mg/kg prednisolone from Day 0-45. All cats were exposed to an exogenous ovarian control regime consisting of 0.088 mg/kg/day progesterone (Day 0 – 37), 75 IU eCG (Day 40) to stimulate follicular growth, and 50 IU hCG (Day 44) to induce ovulation. The cats were ovariohysterectomised on Day 45. Day 30 values were considered baseline as this allowed sufficient time (Days 0-30) for existing (i.e., Day 0) luteal phases to have ceased and the progesterone treatment to completely suppress follicular growth. Linear mixed models indicated that there was no treatment by time interaction for either oestradiol or progesterone concentrations, thus treatment and control groups were compared for each day. †P<0.10, *P<0.05.

4.3.2 Ovarian and oocyte assessments

The effect of exogenous gonadotropin stimulation on ovarian morphology, follicular stimulation, and ovulation did not differ between treatment and control animals, thus data were combined for further analysis. The gonadotrophin treatments successfully stimulated follicular growth and ovulation in all 12 cats, with a mean number of ovulations (determined from number of CH/CL) of 10.5 ± 1.1 (range:

6 – 22). The mean number of large follicles (>2 mm) present per cat was 0.8 ± 0.3. Follicles, CH and/or CL were present on both ovaries of all cats except for Kanuka, in which ovarian structures developed in only one ovary. Kanuka also appeared to have ovulated earlier than the other cats, as indicated by the presence of mature CL at ovariectomy (c.f., only CH in other cats). The number of ovulations per ovary was positively correlated with ovary weight (rho=0.71, P<0.001) and volume (rho=0.69, P<0.001).

The mean ovarian response grade to the gonadotrophin treatments was 1.5 ± 0.4. The oocyte recovery rate was 47.6 ± 9.7 % and oocytes were not recovered from two of the 12 cats (one treatment cat and one control cat). Ocyte recovery rates did not differ between control and treatment animals. A total of 28 and 30 oocytes were collected from the control and treatment cats, respectively.

The oocyte diameter was not influenced by treatment, with a mean oocyte diameter of 54.4 ± 0.8 μm (range: 42.4 – 73.8 μm). The ZP was thinner in the treatment cats (3.1 ± 0.3 μm) than the control (4.1 ± 0.3 μm) cats (P=0.03). Zona pellucida thickness variation was similar between the treatment (46.3 ± 6.1%) and control (39.4 ± 4.2%) groups. The TOS, oocyte morphology grade, and oocyte size grades did not differ between treatments (Figure 4.5). However, there was a tendency for the ZP grade to be lower in the cats receiving prednisolone (P=0.08; Figure 4.5). The ooplasm grade was lower in treatment cats (P=0.01; Figure 4.5**)**.

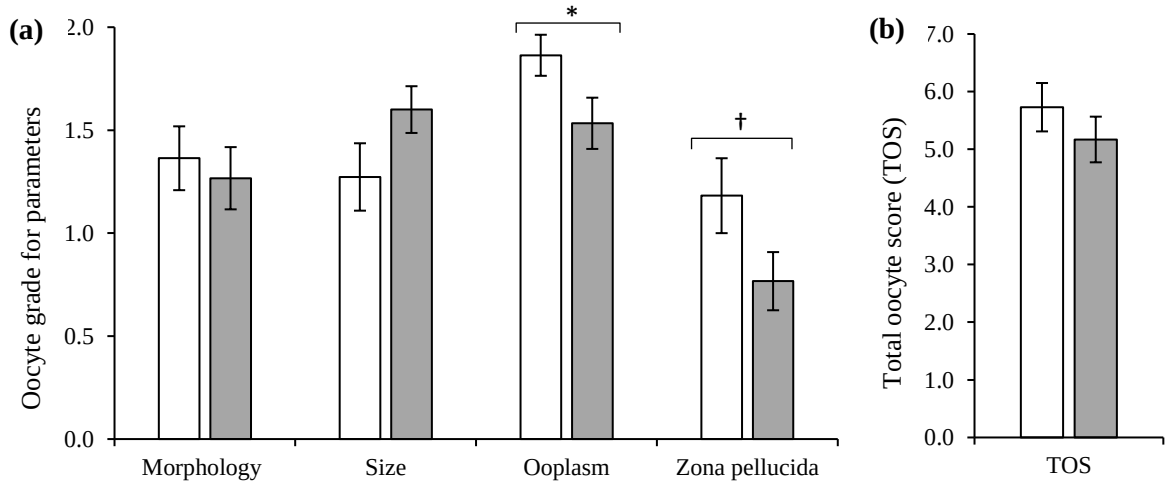

Figure 4.5 (a) Mean (±SEM) oocyte morphology, oocyte size, ooplasm, and ZP grades (0 – 2, 2=best) of oocytes the control (white bars; n=28) and treatment cats (grey bars; n=30) oocytes, with treatment cats given 1 mg/kg/day prednisolone for 45 days. These grades were summed to provide the total oocyte score (b; TOS; 0-8, 8=best). All cats were exposed to an exogenous ovarian control regime consisting of 0.088 mg/kg/day progesterone (Day 0 – 37), 75 IU eCG (Day 40) to stimulate follicular growth, and 50 IU hCG (Day 44) to induce ovulation. †P<0.10, *P<0.05.

A higher proportion of poor quality (TOS=0-3) oocytes was retrieved from treatment cats compared to control animals (P=0.04; Table 4.2). Treatment cats also had lower proportion of good quality (TOS=7-8) oocytes (Table 4.2), although this difference was not significant. The proportion of moderate (TOS=4-6) oocytes was similar between the treatment and control groups (Table 4.2). There were fewer poor-quality oocytes than both moderate and good quality oocytes for both the treatment and control cats (P<0.05; Table 4.2). The number of moderate and good quality oocytes did not differ between the treatment and control cats.

Table 4.2 The percentage of poor, moderate, and good quality oocytes retrieved from untreated control cats (n=6 cats and n=28 oocytes) and cats treated with 1 mg/kg prednisolone for 45 days (n=6 cats and n=30 oocytes). All cats were exposed to an exogenous ovarian control regime consisting of 0.088 mg/kg/day progesterone (Day 0 – 37), 75 IU eCG (Day 40) to stimulate follicular growth, and 50 IU hCG (Day 44) to induce ovulation. Oocytes were graded on four parameters (morphology, size, ooplasm, and ZP), with each parameter being graded from 0 – 2 (2=best). The total oocyte score (TOS) was the sum of the grades for the four oocyte parameters. P>0.10 reported as non-significant (NS).

Oocyte quality (TOS)	Percentage of oocytes		P-value
	Control	Treatment	
Poor (0-3)	13.6	26.7	0.04
Moderate (4-6)	40.9	40.0	NS
Good (6-8)	45.5	33.3	NS

4.4 Discussion

This is the first study to examine the effects of exogenous GC treatment (i.e., a simulated endocrine stress response) on the ovarian response of domestic cats to the exogenous stimulation of follicular growth and ovulation. The hypothesis that GC treatment would have a negative impact on ovarian stimulation, ovulatory response, and oocyte quality was partially supported. The exogenous gonadotrophin treatments successfully induced follicular growth and ovulation in all cats. Body weight, ovarian weight and volume, ovarian response grade, number of ovulations, and oocyte recovery did not differ between prednisolone-treated and control cats. While oocyte diameter was comparable between the treatment and control cats, the ZP was thinner in cats given prednisolone. Oocyte recovery was <50% for both groups and the TOS did not differ between the two groups of cats, but ooplasm grade was lower in the treatment group. There was also a higher proportion of poor-quality oocytes recovered from cats in the treatment group.

Prednisolone treatment did not appear to affect the circulating cortisol and glucose concentrations of female cats undergoing exogenous gonadotropin treatment. This was surprising given that exogenous GC treatments are known to decrease endogenous cortisol concentration and increase glucose concentrations (Middleton *et al.*, 1987; Kuo *et al.*, 2015). Prednisolone and prednisone were undetectable in the serum of both prednisolone-treated and control animals. As mentioned in Chapter 3, there is conflicting data on the half-life of prednisolone, with veterinary textbooks reporting a 12-36

hour half-life (Behrend and Kemppainen, 1997; Feldman and Nelson, 2004) and a human pharmacokinetic study reporting a 2-3 hour half-life (Rose *et al.*, 1981). If the prednisolone half-life is in fact 2-3 hours, then it is likely that circulating prednisolone concentrations were below the limit of quantification (i.e., <0.25 ng/mL) by the time sampling occurred, which was 22-24 hours after the prednisolone treatment.

The progesterone concentrations measured herein were similar to those reported in past studies, which reported baseline and luteal (i.e., dioestrus) progesterone concentrations of 0.7 ng/mL (range: <0.1-3.1 ng/mL) and 39.1 ng/mL (2.1-187 ng/mL), respectively (Paape *et al.*, 1975; Verhage *et al.*, 1976; Shille *et al.*, 1979; Shille and Stabenfeldt, 1979; Schmidt *et al.*, 1983; Goodrowe *et al.*, 1988b; Chatdarong *et al.*, 2006; Chatdarong *et al.*, 2007). While progesterone concentrations were lower in prednisolone-treated cats than control cats one day after the hCG treatment (i.e., Day 44), the physiological significance of this unclear. Baseline serum oestradiol concentrations measured in the present study were higher than previously described in cats (Verhage *et al.*, 1976; Shille *et al.*, 1979; Donoghue *et al.*, 1992; Swanson *et al.*, 1997). It has been reported that baseline serum or plasma oestradiol concentrations are ~11 pg/mL (range: 1.1-20; Verhage *et al.*, 1976; Shille *et al.*, 1979; Donoghue *et al.*, 1992; Swanson *et al.*, 1997), but in the present study baseline oestradiol concentrations were 40.7 ± 5.1 pg/mL. These past studies examining serum oestradiol concentrations in cats are now >20 years old (Verhage *et al.*, 1976; Shille *et al.*, 1979; Donoghue *et al.*, 1992; Swanson *et al.*, 1997), thus it is possible that assay specificity and oestradiol recovery has been improved in modern assays. Regardless, the discrepancy between the oestradiol concentrations observed in this study and reported in past literature reinforces the need for baseline and peak oestradiol comparison for each study and perhaps each animal, as one cat had substantially higher oestradiol concentrations than the others (baseline = 83 pg/mL and peak = 327 pg/mL) in the present study.

The exogenous progestin treatment successfully suppressed ovarian function of all cats. Stewart *et al.* (2010) found that 0.088 mg/kg/day altrenogest was optimal for follicular suppression in cats, which was supported by the results of this study. It has been suggested that the duration of progesterone treatment needs to encompass an entire non-pregnant luteal phase, which is 40 (range: 26-55) days (Pelican *et al.*, 2008; Andrews *et al.*, 2019). As reported previously, the progesterone treatment does not affect the duration or amplitude of existing luteal phases and cannot override existing follicular waves (Pelican *et al.*, 2005; Pelican *et al.*, 2010; Stewart *et al.*, 2010), thus the treatment period needs to be longer than a potential follicular and/or luteal phases. In the present study, 30 days was sufficient for all luteal phases (n=2) to have concluded and all follicular development to be suppressed. Thus, the present study and past literature indicates that 37-38 days is a sufficient duration of progesterone treatment to cause complete ovarian quiescence (Pelican *et al.*, 2008; Stewart *et al.*, 2010; Stewart *et al.*, 2012).

Progesterone priming has been found to enable the cat ovary to respond consistently to relatively low concentrations of eCG and hCG, which is important as high gonadotrophin doses have been shown to adversely affect luteal function (i.e., abnormally high progesterone concentrations) and oocyte/embryo quality in cats (Roth *et al.*, 1997b; Graham *et al.*, 2004; Pelican *et al.*, 2006; Stewart *et al.*, 2010; Stewart *et al.*, 2012). Stewart *et al.* (2012) found that the optimal doses of eCG and hCG in cats following progestin pre-treatment were 50 IU eCG and 37.5 IU hCG, respectively. However, in one study that used this regime, two out of eight cats failed to ovulate (Stewart *et al.*, 2015). In the present study, the slightly higher doses of 75 IU eCG and 50 IU hCG were sufficient to induced follicular growth and development in all 12 cats; thus, these doses may be more appropriate.

The prednisolone treatment did not appear to affect the number of ovulations per cat. The number of ovulations observed in this study (10.5 ± 1.1 ovulations per cat, range: 6 – 22) is comparable to other studies that have induced follicular growth and ovulation using exogenous gonadotrophins (mean of 9-15 ovulations per cat), but was higher than reported for natural oestrus and ovulation (~5.0 ovulations per cat; Wildt *et al.*, 1981b; Goodrowe *et al.*, 1988b; Donoghue *et al.*, 1993; Swanson *et al.*, 1997; Stewart *et al.*, 2012).

Oocyte recovery was 47.6%. It is unclear how effective the oocyte retrieval method used here was because to my knowledge there are no comparative data available on oviductal oocyte recovery rates in cats. The majority of studies have aspirated oocytes from ovarian follicles *in vivo* or following ovariohysterectomy (Goodrowe *et al.*, 1988b; Roth *et al.*, 1994; Wood and Wildt, 1997; Comizzoli *et al.*, 2003; Pelican *et al.*, 2006; Pope *et al.*, 2006; Pelican *et al.*, 2010; Pope *et al.*, 2012; Pope, 2014). To the best of my knowledge, the most comparable study in cats was Graham *et al.* (2000), in which oocytes/embryos were retrieved 160 hours after an hCG-induced ovulation and a 52.5% embryo recovery rate (based on the number of corpora lutea) reported. Oocyte or embryo retrieval following uterine flushing has resulted in similar recovery rates to the present study in other species such as dogs (*Canis familiari*; 62.5% embryo recovery; Jeong *et al.*, 2016), goats (*Capra aegagrus*; 43.0% embryo recover; Suyadi and Holtz, 2000), and sheep (*Ovis aries*; 50.0% oocyte recovery; Flohr *et al.*, 1999). Thus, while the oocyte recovery rate appeared to be low in the present study, it was comparable to past studies. Furthermore, there was no evidence that the prednisolone treatment affected oocyte recovery rates.

The GC treatment did not reduce the TOS of feline oocytes, but there was higher proportion of poor-quality oocytes recovered from cats given prednisolone. In addition, ZP was thinner and there was a tendency for ZP graded to be lower in prednisolone-treated cats than control cats. The literature suggests that ZPTV is more important than ZP thickness in the context of *in vitro* fertilisation success, at least in humans (Palmstierna *et al.*, 1998; Gabrielsen *et al.*, 2001; Sun *et al.*, 2005). The prednisolone treatment administered herein did not appear to affect the ZPTV, but the ooplasm grade

was slightly lower for treated cats. Oocytes that have a darkened or granulated cytoplasm have been found to have reduced developmental competence in humans (Veeck, 1988; Serhal *et al.*, 1997; Kahraman *et al.*, 2000). Granularity is normally considered indicative of cytoplasmic immaturity; thus, it is possible the GC treatment affected oocyte maturation. Indeed, corticosterone has been shown to adversely affect both the cytoplasmic and nuclear maturation of mouse oocytes *in vitro* (González *et al.*, 2010). The impaired oocyte maturation following GC treatment has been linked to reduced activity of mitogen activated protein kinases (MAPK) in mouse oocytes *in vitro* (González *et al.*, 2010).

Corticotrophin-releasing hormone and GC appear to impair cytoplasmic maturation and increase apoptosis in cumulus cells and mural granulosa cells *in vitro* (Liang *et al.*, 2013; Yuan *et al.*, 2020). The administration of a physiological dose of exogenous cortisol (i.e., to simulate the increase in cortisol concentrations associated with restraint stress) has been found to reduce oocyte quality and to induce apoptosis in ovarian cells in mice (Yuan *et al.*, 2016; Yuan *et al.*, 2020). The pathways by which GC induce apoptosis in the ovary are not clear, but have been linked to an increase in both tumour necrosis factor α (TNF-α) and Fas/FasL expression (Yuan *et al.*, 2016; Yuan *et al.*, 2020). Irrespective of the pathways involved, the concept that the amount of apoptotic cumulus and mural granulosa cells is negatively correlated with the developmental competence of the oocyte is fairly well supported (Lee *et al.*, 2001; Wang and Sun, 2006; Liang *et al.*, 2013; Yuan *et al.*, 2020). It would be interesting to determine whether prednisolone treatment increased the apoptosis of cells within the cat ovary in the present study, since ovarian tissue was collected.

The effects of elevated GC on oocyte development and ovarian function of cats could be minimised through exogenous stimulation of follicular growth and ovulation. This is promising in the context of ART for captive non-domestic felids, as increases in GC concentrations associated with captivity-related stress would likely have a minimal effect on ovulation rates and oocyte quality following eCG/hCG administration. In the future, it would be worthwhile examining whether GC administration affects ovarian function and oocyte quality during a natural oestrous cycle in cats. As mentioned previously, elevated GC concentrations associated with captivity have been associated with ovarian quiescence in cheetah (Jurke *et al.*, 1997), margay (*Leopardus weidii*), and trigrina (*Leopardis tigrinus*; Moreira *et al.*, 2007). However, there has been no direct physiological link between elevated GC and reduced oestrous cyclicity in felids. A study that monitored the natural ovarian cyclicity of cats treated with GC would be valuable, and it would also be interesting to collect ovaries and oocytes from naturally mated cats given exogenous GC.

4.5 Conclusions

In conclusion, progestin-mediated follicular suppression followed by eCG and hCG stimulation resulted in follicular growth and ovulation in all cats. A prednisolone treatment intended to simulate

an endocrine stress response had little effect on the response of felid ovaries to exogenous gonadotrophin treatments, with number of ovulations being similar between treatment and control cats. The prednisolone treatment did not appear to affect the TOS, but cats given prednisolone had a higher proportion of poor quality oocytes and lower ooplasm and ZP scores. Whether this affects fertility remains to be determined. In the future, it would be worth investigating whether GC treatments alter the fertilisation capabilities of cat oocytes. It is possible that the stimulation of follicular growth and ovulation with exogenous gonadotrophins mitigated the effects of prednisolone. Thus, it would be also be valuable to assess the effects of GC administration on the natural oestrous cyclicity of domestic cats.

Chapter 5

Validation of Heyrex® accelerometers for monitoring the activity and behaviour of domestic cats (*Felis catus*)

Heyrex® activity monitor. Image taken from www.heyex.com.

Chapter 5: Validation of Heyrex® accelerometers for monitoring the activity and behaviour of domestic cats (*Felis catus*)

5.0 Abstract

Accelerometry allows quantification of the activity and behaviour of animals without time-consuming behavioural observations. Heyrex® devices wirelessly upload data onto a server that can be accessed remotely, thus enabling the real-time monitoring of behaviour. This study aimed to develop and validate Heyrex® devices (designed for dogs) for remotely assessing the behaviour of domestic cats (*Felis catus*). Heyrex® devices were fitted to the existing collars of five cats. Heyrex® and observed behavioural data were collected concurrently for 24 hours and compared. Observed and Heyrex® overall activity counts were highly correlated for all five cats (mean ± SEM: rho 0.94 ± 2.2; $P<0.001$). Based on observed data, the cats spent 47.9 ± 5.6% (mean ± SEM) of their time sleeping, 37.7 ± 5.3% resting, 6.9 ± 1.9% walking, 4.5 ± 2.0% grooming, 1.7 ± 0.3% eating, 1.1 ± 0.5% drinking, and 0.1 ± 0.05% running. The categorisation of walking behaviour by the Heyrex® devices was inconsistent, with the observed time spent walking being positively correlated with Heyrex®-recorded time spent walking (rho = 0.90; $P<0.001$), resting (rho = 0.81; $P<0.001$), and running (rho = 0.60; $P<0.001$). The Heyrex® devices possibly miscategorised walking behaviour, because the canine-designed algorithms were not sensitive enough for the more subtle walking movements of cats. Scratching behaviour was also poorly categorised by the Heyrex® devices. The observed time spent scratching was positively correlated with the Heyrex®-recorded time spent resting (rho = 0.42; $P<0.001$), scratching (rho = 0.44; $P<0.001$), and walking (rho = 0.47; $P<0.001$). Observed eating, grooming, and drinking were moderately correlated ($P<0.001$) with both the time spent resting (rho = 0.37, 0.55, 0.33, respectively) and walking (rho = 0.50, 0.48, 0.45, respectively), as recorded by the Heyrex®. Cats spent a noteworthy amount of time grooming, thus the incorporation of grooming into the Heyrex® behavioural algorithms is advisable. Compared to other accelerometer-based devices used on cats, there appeared to be much less variation in the relationship between the Heyrex® and observed overall activity counts of different cats. Other modification (e.g., size or shape) of the devices may also be required to optimise their use for domestic cats.

5.1 Introduction

Accelerometry offers a means of remotely quantifying the activity and behaviour of animals without the need for direct and often time-consuming behavioural observations. To date, accelerometers have been used to accurately monitor the activity of a wide variety of animals including cattle (*Bos Taurus*; McGowan *et al.*, 2007), cheetah (*Acinonyx jubatus*; Watanabe *et al.*, 2005; Grünewälder *et al.*, 2012), koala (*Phascolarctos cinereus*; Takahashi *et al.*, 2009), badgers (*Meles meles*; McClune *et al.*, 2014), elephants (Rothwell *et al.*, 2011), rhesus monkeys (*Macaca mulatta*; *et al.*, 2007; Papailiou *et al.*, 2008), humans (*Homo sapiens*; Trost *et al.*, 2000; Hartel *et al.*, 2011), dogs (*Canis familiaris*; Brown

et al., 2010), and cats (*Felis catus*; Watanabe *et al.*, 2005; Lascelles *et al.*, 2007; Andrews, 2015; Andrews *et al.*, 2015). In companion animals such as cats and dogs, accelerometry has mainly been used for the identification and monitoring of conditions such as osteoarthritis (Lascelles *et al.*, 2007; Brown *et al.*, 2010).

Actical® (MiniMitter, Bend, OR, USA) accelerometers were used initially to assess the efficacy of non-steroidal anti-inflammatory drugs for treating joint diseases in cats and dogs, eliminating the need for unreliable owner-based assessments (Lascelles *et al.*, 2001; Lascelles *et al.*, 2007; Brown *et al.*, 2010). As the most common recommendation for animals with joint disease is weight loss (Crane, 1991; Lascelles, 2010), accelerometry has also been used to investigate various feeding regimes that could be used to combat obesity in companion animals (Alexander *et al.*, 2014; Deng *et al.*, 2014). Accelerometers have also been investigated for assessing other conditions such as reproductive state (Andrews, 2015), identification of stress behaviour in kennels/catteries (Jones *et al.*, 2014), and monitoring the effects of various medical treatments and post-operative recovery (Delgado *et al.*, 2014; Helm *et al.*, 2016; Little *et al.*, 2016).

Despite the numerous applications of accelerometry, most accelerometer-based devices have limitations, the most concerning being the considerable amount of inter-individual variation between accelerometer outputs and observed activity or behaviour (Lascelles *et al.*, 2008; Andrews *et al.*, 2015). In addition, most commercially available accelerometer-based devices for animals only provide information on overall activity and lack algorithms for identifying the expression of specific behaviours. Lastly, most devices need to be removed to extract their data, which is limiting in a clinical context as it requires the owner to bring the device or animal into the clinic.

Heyrex® activity monitors (Heyrex® Ltd., Wellington, New Zealand) wirelessly upload data onto an online server that can be accessed remotely, and they offer the longest battery-life (~2 years) of all commercially available accelerometers for animals to date. Heyrex® activity monitors have been specifically developed for dogs, with the devices accurately determining the overall physical activity (OPA) and the expression of a range of behaviours (e.g., sleeping, resting, walking, running; Edwards and Gibson, 2012; Mejia *et al.*, 2019). Given the advantages offered by the Heyrex® monitors, the adaptation of these devices for use on domestic cats would provide an accurate and reliable tool for monitoring the activity and behaviour of cats. This study therefore aims to validate Heyrex® activity monitors for remotely assessing the activity and behaviour of domestic cats. The specific objective was to compare and correlate the activity and behaviour data provided by the Heyrex® monitors against that obtained by visual observation of the cats.

5.2 Materials and methods

5.2.1 Animals

Five healthy, de-sexed domestic cats (three female and two male) in the Massey University Centre for Feline Nutrition were used for this study. The cats ranged in age from two to eight years (mean ± SD, 6.50 ± 1.03 years) and were housed in a mixed-sex group of eight. The cats had *ad libitum* access to water and were fed a complete and balanced commercial wet food diet (Kraft-Heinz Wattie's Ltd, Hastings, New Zealand; AAFCO, 2020). All research was conducted in accordance with Massey University Animal Ethics Committee protocol number 18/07.

5.2.2 Experimental design

Heyrex® devices (Heyrex Ltd., Wellington, New Zealand) were attached to the existing collars of the cats (Figure 5.1). The devices measured approximately 65 mm x 25 mm x 10 mm and weighed 20 g. The cats were given one week to acclimitise to the devices before data collection. The cats were housed in their normal pens, which were under continuous video surveillance using a TechView H.264 digital video security camera system (Tech Brands: Electus Distribution, Auckland, New Zealand). Video footage and Heyrex® data were collected concurrently for 24 hours.

Figure 5.1 Cat wearing Heyrex® activity monitor.

5.2.3 Assessment of activity and behaviour

The observed behaviour of the cats was assessed retrospectively from video footage. Continuous duration sampling was used to determine the amount of time each cat spent sleeping, resting, grooming, eating, drinking, scratching, walking, and running over a 24-hour period (assessed at 5 minute intervals). Observed OPA was determined by categorising behaviours as either active (walking, running, scratching, playing, climbing, and grooming) or inactive (eating, drinking, sleeping, and resting).

The Heyrex® devices use tri-axial accelerometry (i.e., measurement of acceleration along three independent axes: forward-backwards, left-right, and up-down) to quantify activity as the change in acceleration from gravity ($9.8 m/s^2$; ΔG). A canine-based behavioural algorithm (Canine V1) was used to determine the duration and frequency of walking, running, scratching, sleeping, and resting. The activity monitors recorded the ΔG (a single measure of intensity, frequency, and duration) and amount of time (seconds) that the cats spent displaying walking, running, scratching, sleeping, and resting behaviours at 5-minute intervals (or epochs). Overall physical activity was calculated as the sum of the ΔG values for each behaviour, defined as total ΔG (TΔG).

5.2.4 Statistics

Accelerometry activity and behaviour data (5-minute epochs) were summed to provide the TΔG for each behaviour and time spent exhibiting each behaviour per hour. Concurrent observed activity and behaviour data were also summed to provide hourly totals. A Shapiro-Wilk test was used to determine the normality of the data. The data were non-parametric, thus Spearman-rank correlation tests were used to determine the relationship between the observed and Heyrex® data for each cat, and for all cats combined. Correlation coefficients for individual behaviours were categorised as very weak (rho <0.10), weak (rho 0.10<0.30, moderate (rho 0.30<0.60) or strong (rho >0.60). All analyses were conducted using RStudio 1.0.143 (R Foundation for Statistical Computing, Vienna, Austria) with a significance level of $P<0.05$. Values are presented as mean ± SEM unless otherwise indicated.

5.2 Results

A total of 120 hours of concurrent Heyrex® and observed activity/behaviour data were collected from the five cats. The average percentage of time that the cats were observed to be active (excluding grooming) over the 24 hour period was 7.1 ± 1.9% (range; 2.2-13.9%).

The TΔG outputs from the Heyrex® devices accurately represented the observed OPA of the cats at five-minute intervals over 24 hours (rho=0.82, $P<0.001$). However, increasing the sampling interval to one hour increased the strength of the correlation between TΔG and the observed activity counts of the cats (rho = 0.94, $P<0.001$). Thus, all OPA assessments hereafter were conducted using the one-hour epoch data. The TΔG and observed activity counts of individual cats were highly correlated (rho = 0.94 ± 0.02, $P<0.001$). The relationships between TΔG and observed activity were similar among

cats, except for one cat (Coby; Figure 5.2). This animal spent more time grooming (12.5% of the time) than the other cats (2.5±0.5%, range: 1.3-3.5%), and the incorporation of grooming into the observed activity counts of Coby resulted in the regression changing considerably with respect to the other cats (Figure 5.2).

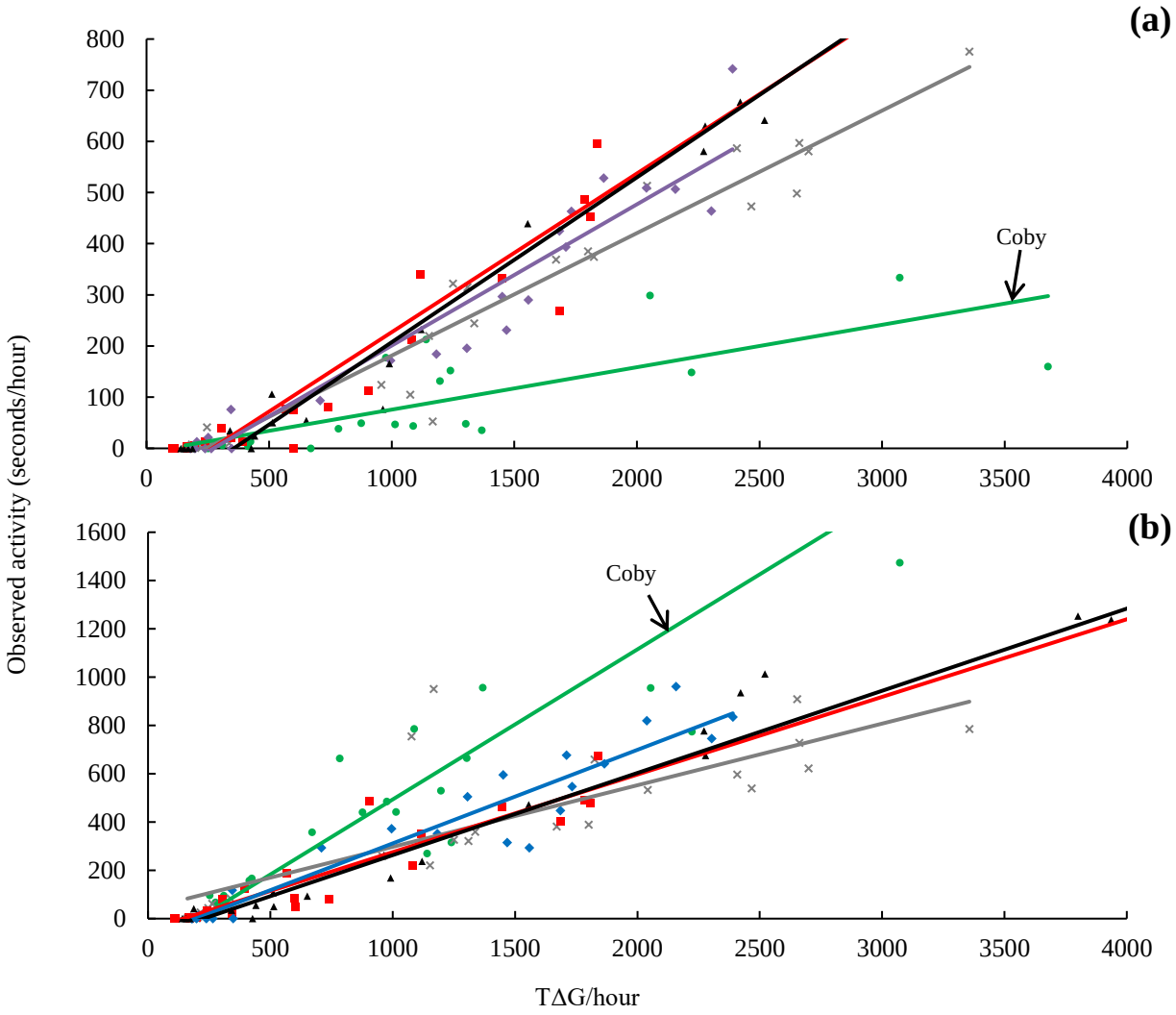

Figure 5.2 The regression analyses between the total Heyrex® activity counts (TΔG) and observed activity of the cats (a) excluding grooming and (b) including grooming. Note the effect of adding grooming behaviour to the correlation between Total ΔG and observed activity for Coby.

Based on observed data, the cats spent on average 47.9 ± 5.6% (range: 28.1-61.2%) of the time sleeping, 37.7 ± 5.3% (range: 30.3-58.7) resting, 6.9 ± 1.9% (range: 2.0-13.7%) walking, 4.5 ± 2.0% (range: 1.3-12.3%) grooming, 1.7 ± 0.3% (range: 0.8-2.8%) eating, 1.1 ± 0.5% (range: 0.3-2.6%) drinking, and 0.1 ± 0.05% (range: 0.03-0.3%) running. The behavioural outputs (sleeping, resting, scratching, walking, and running) from the Heyrex® devices were significantly correlated with

observed behaviours (P<0.001 for all behaviours). Increasing the sampling interval (5 minutes vs 1 hour) increased the correlation coefficients between Heyrex® and observed behavioural assessments for all behaviours but was only significant for walking behaviour (Figure 5.3). The mean spearman correlation coefficients between observed behaviour and the Heyrex® outputs (1 hour epoch) were: sleeping (rho = 0.93 ± 0.03; P<0.001), resting (rho = 0.86 ± 0.05; P<0.001), scratching (rho = 0.36 ± 0.11; P<0.001), walking (rho = 0.92 ± 0.36; P<0.001), and running (rho = 0.55 ± 0.08 P<0.001). Spearman correlation coefficients were lower for running and scratching behaviours (Figure 5.3), with the correlation between the Heyrex® and observed assessments of running and scratching being only significant for one and two cats, respectively. The relationship between Heyrex® and observed behaviour were highly similar for sleeping and resting behaviour, but varied considerably between cats for walking and running behaviour (Figure 5.4).

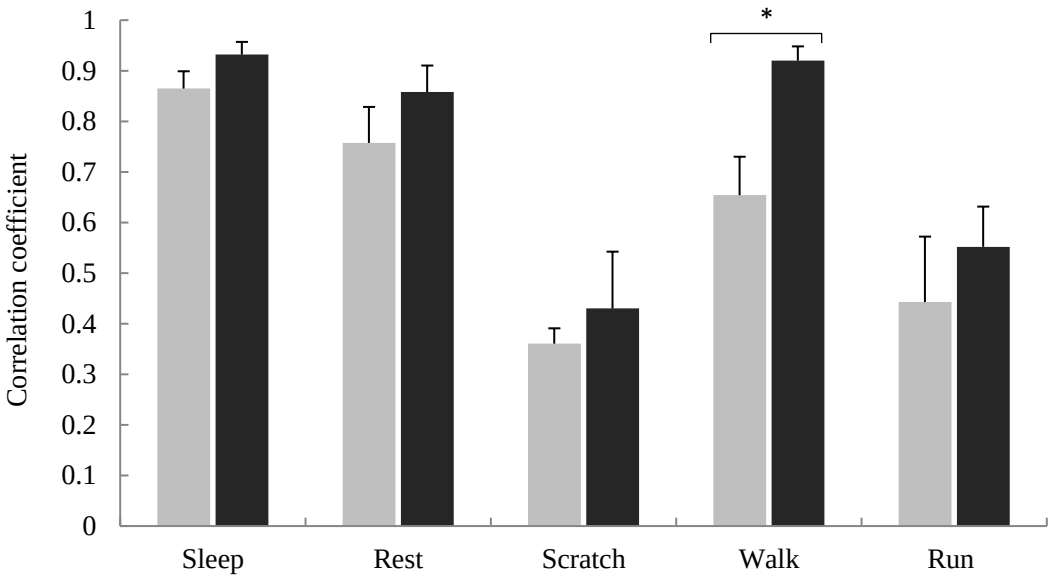

Figure 5.3 Spearman correlation coefficients between the Heyrex and observed behavioural assessments of five cats at five minute (grey) and 1 hour (black) intervals over 24 hours. Note that data on scratching behaviour were only available for four cats as one cat did not exhibit this beahviour. *P<0.05.

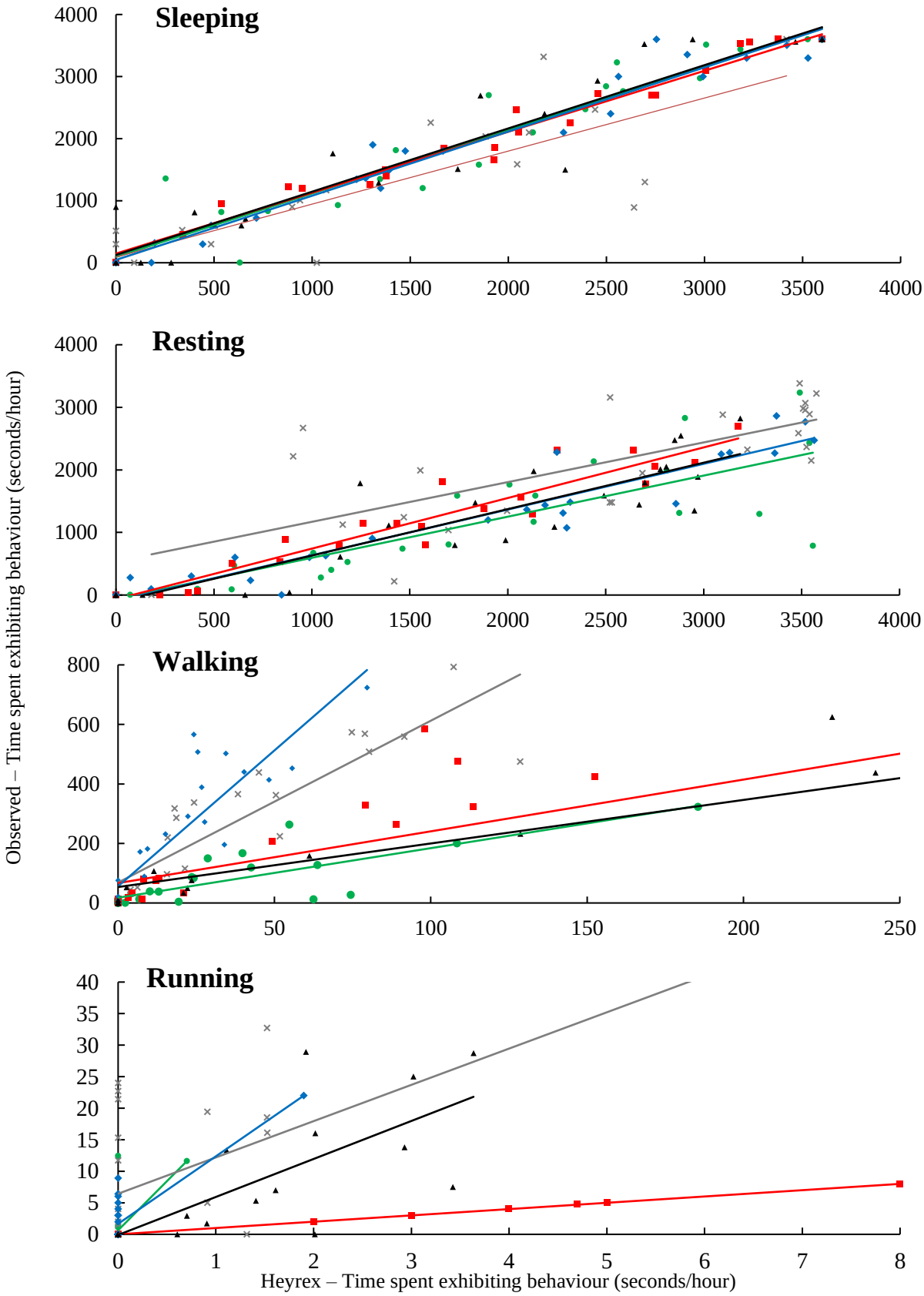

Figure 5.4 The regression analyses between Heyrex® and observed time (seconds) spent exhibiting sleeping, resting, walking, and running behaviour per hour over a 24 hour period for the five cats.

There was cross-correlation among the different behaviours (Table 5.1). While Heyrex®-recorded time spent resting per hour had a strong positive correlated with observed time spent resting (rho = 0.87; *P*<0.001), it was also positively correlated with observed time spent scratching (rho = 0.42; *P*<0.001), walking (rho = 0.81; *P*<0.001), running (rho = 0.59; *P*<0.001), grooming (rho = 0.55; *P*<0.001), eating (rho = 0.37; *P*<0.001), and drinking (rho = 0.33; *P*<0.001; Table 5.1). The categorisation of walking behaviour by the Heyrex® devices was inconsistent, with the observed time spent walking having a strong positive correlation with not only Heyrex®-recorded time spent walking (rho = 0.90; *P*<0.001), but also time spent resting (rho = 0.81; *P*<0.001) and running (rho = 0.60; *P*<0.001; Table 5.1). Observed time spent scratching was positively correlated with Heyrex®-recorded resting (rho = 0.42; *P*<0.001), scratching (rho = 0.44; *P*<0.001), and walking (rho = 0.47; *P*<0.001) behaviours (Table 5.1). Furthermore, observed time spent eating, grooming, and drinking were moderately correlated (*P*<0.001 for all comparisons) with both the time spent resting (rho = 0.55, 0.37, 0.33, respectively) and walking (rho = 0.48, 050, 0.45, respectively), as recorded by the Heyrex® (Table 5.1).

Table 5.1 Correlations between the Heyrex® and observed time spent exhibiting sleeping, resting, scratching, walking, and running behaviour per five minutes over a 24-hour period. Spearman correlation coefficients were defined as strong (rho>0.60), moderate (rho = 0.30-0.60), weak (rho<0.30), or very weak (rho<0.10). Very weak and negative correlation coefficients are no shown. Behaviours that were exhibited by the cats but were not among the behaviours assessed by the Heyrex® devices were defined as 'observed (other)'. Grey cells indicate target correlations. Red and orange cells indicate unexpected strong or moderate positive correlations, respectively.

		Observed					Observed (other)		
		Sleep	Rest	Scratch	Walk	Run	Groom	Eat	Drink
Heyrex	% time exhibiting behaviour/24h	47.92	37.74	0.10	6.91	0.13	4.49	1.67	1.08
Sleep	45.50	Strong							
Rest	51.92		Strong	Moderate	Strong	Moderate	Moderate	Moderate	Moderate
Scratch	0.01			Moderate	Weak	Weak	Weak	Weak	
Walk	2.52		Moderate	Moderate	Strong	Strong	Moderate	Moderate	Moderate
Run	0.01		Weak	Weak	Strong	Moderate		Weak	Weak

5.3 Discussion

The present study investigated whether Heyrex® devices could accurately quantify the overall activity and specific behaviours of the cats as compared to visual observation and quantification. Heyrex® and observed activity counts were highly correlated. Furthermore, the relationships between the Heyrex® and observed activity counts of different cats were consistent. The Heyrex® devices also provided an accurate representation of sleeping and resting behaviour, with little inter-cat variation. However, the devices were less consistent among cats in distinguishing walking and running behaviour. In fact, there was considerable variation in the relationships between the Heyrex® and observed time spent walking and running for different cats.

The between the Heyrex® and observed activity total counts in cats are similar to those reported for other accelerometer devices such as Actical® accelerometers (Lascelles *et al.*, 2007; Andrews *et al.*, 2015). Previous studies have indicated that caution is needed when interpreting accelerometer-based data for research in cats due to high inter-cat variation (Lascelles *et al.*, 2007; Andrews *et al.*, 2015). In fact, it has been stated that the inter-cat variation is so significant, that each animal should be considered to be its own control and the activity data of different animals should not be compared (Andrews *et al.*, 2015). Interestingly, the regression lines between the Heyrex and observed activity data of different cats were similar (excluding coby; Figure 5.2), suggesting that the devices were more consistent among cats. However, there was considerable variation among cats for specific behaviours.

The Heyrex® devices frequently miscategorised resting, scratching, walking, and running behaviour (Table 5.1). The inaccuracy of the behavioural assessments of the Heyrex® devices likely rest on the behavioural algorithms used, which were developed for canine behaviour. The movement and behaviour of dogs is more linear and less complex than cats (Gahery *et al.*, 1980; O'Farrell, 1992; Bradshaw, 2012). For example, cats exhibit vertical climbing behaviour while dogs do not, and cats also exhibit considerably more grooming behaviour than dogs (O'Farrell, 1992; Bradshaw, 2012). Furthermore, during locomotion the cat's head is very stable and exhibits very little vertical or horizontal movement. This may be why the collar-attached Heyrex® activity monitors struggled to distinguish between resting and low intensity locomotor behaviour: walking and running were identified by Heyrex® behavioural algorithms as resting, and Heyrex®-assessments of resting behaviour were positively correlated with both the observed time spent walking and running. Consequently, it seems that the existing algorithms used by the Heyrex® devices to assess walking and running behaviour need to be modified to increase sensitivity for cats.

Grooming behaviour adversely affected the accuracy of the Heyrex® devices for detecting other behaviours, since these behaviours were associated with increases in the Heyrex® counts for resting, walking, and running behaviour. The effect of grooming has been previously shown to adversely affect the accuracy of accelerometer devices (Andrews *et al.*, 2015). Grooming behaviour is often vigorous and requires considerable movement and stretching, and cats spend a considerable proportion of their time grooming (~8%; Eckstein and Hart, 2000b), although lower levels than this were observed in the present study in all but one cat (~4.5%). The incorporation of grooming behaviour into the assessments of Heyrex® devices would be beneficial for various applications such as to study the efficacy of NSAIDs for the treatment of joint disease (Lascelles *et al.*, 2007; Lascelles, 2010), since grooming may be indicative of effective pain therapy (Hardie, 1997; Lascelles, 2010). Furthermore, excessively grooming can be indicative of stress (van den Bos, 1998), oestrus (grooming and allogrooming is increased during oestrus), and fleas or other skin irritations (Eckstein and Hart, 2000a; b). Conversely, low levels of grooming results in poorer coat condition and may be associated with illness or high levels of stress (Odendaal, 1997; Tanaka *et al.*, 2012; Uetake *et al.*, 2013).

When using other devices (e.g., Actical® accelerometers), it has been suggested that inter-cat variation is due to differences in behaviour (e.g., more grooming) or differing collar tightness affecting the residual movement of the devices (Andrews *et al.*, 2015). The Heyrex® devices are larger (65 mm x 25 mm x 10 mm) and attach more tightly to the cats' collar than Actical® accelerometers (28 mm × 27 mm × 10 mm), which could reduce the residual movement of the devices in response to various behaviours (e.g., walking, running, grooming and scratching). The current Heyrex® devices appeared large on domestic cats, especially smaller cats (i.e., less than 3 kg.). While it may seem appealing to reduce the size of the devices to better suit cats, it is possible that doing this may increase inter-cat variation due to greater residual movement of the devices (as seen with Actical® accelerometers; Andrews *et al.*, 2015). According to Coughlin and van Heezik (2015), cats should not have devices that weigh greater than 2% of their body weight attached to their collars. Heyrex® accelerometers are well below this weight (e.g., less than 1% of the bodyweight of cats that are greater than 2 kg) despite being large in terms of dimensions. It would be worthwhile comparing the accuracy of the existing and smaller devices for monitoring the behaviour of cats, although the algorithms should be modified for cats before doing this.

This study was conceived as part of an on-going association with Heyrex Ltd. to adapt and develop the Heyrex® devices for monitoring the activity and behaviour of cats. This included hardware and algorithm modifications, and validation based on detailed observational data. Unfortunately, the developmental phase of this study could not be continued as Heyrex Ltd. ceased commercial activities in 2019. Furthermore, when Heyrex® Ltd. closed down their online servers stopped working, thus, activity data can no longer be collected from the Heyrex® devices.

5.4 Conclusions

Heyrex® activity monitors designed for dogs provided an accurate assessment of the overall activity of domestic cats. Furthermore, there was little variation in the relationship (regression lines) between the Heyrex® and observed activity counts of different cats when compared to other devices such as Actical® accelerometers. The behavioural assessments of the Heyrex® devices, however, were less accurate. The devices accurately assessed sleep and resting behaviour, but the assessment of walking, running, and scratching behaviour was erratic. The existing algorithms for these active behaviours would need to be modified for use in cats. It is recommended that grooming is considered in the behaviour assessment of the Heyrex®, as grooming is a common behaviour in cats and currently appears to impair the accuracy of the Heyrex® devices for other behaviours. Unfortunately, the development of the Heyrex® devices for cats could not be pursue as Heyrex Ltd ceased activities, but the continuation of this line of research could be of interest for many other companies in the small animal activity monitoring business.

Chapter 6

Accelerometry and infrared thermography show potential for monitoring ovarian function in domestic cats (*Felis catus*)

Chapter 6: Accelerometry and infrared thermography show potential for monitoring ovarian function in domestic cats (*Felis catus*)

6.0 Abstract

Accurate and reliable monitoring of ovarian function is challenging in many felids as current methods are either invasive or not amenable to real-time assessments. This study assessed whether accelerometry and IR thermography can be used to address these limitations. Intact female domestic cats (*n*=12) were given 0.088 mg/kg oral progestin (altrenogest) daily from Day 0 to 37 of the study to suppress follicular growth. On Day 40, cats were given 75 IU equine chorionic gonadotrophin (eCG) to induce follicular growth and 50 IU human chorionic gonadotrophin (hCG) 80 hours later to induce ovulation. Cats were ovariohysterectomised 30-31 hours after the hCG treatment. Cats were fitted with Actical® accelerometers and activity monitored continuously from Day 0 until ovariohysterectomy. Infrared (IR) images of the perivulvar and gluteal area were taken of each cat on study Day 30 and from Days 36 to 45. Perivulvar temperature (PVT), PVT relative to gluteal temperatures (PVT-GT), and PVT relative to perianal temperature (PVT-PAT) were recorded for each image. Blood samples were collected on study Days 0, 10, 30, and 40, prior to the hCG treatment, and at the time of ovariohysterectomy. Changes in serum oestradiol concentrations indicated successful follicular suppression by progestin treatment and, together with morphological assessment of the ovaries, confirmed the induction of follicular growth and ovulation. Daily activity counts differed among cats (P<0.001), thus the daily activity counts of each cat were converted to a proportion of the average daily activity count (proportional daily activity). Proportional daily activity counts were higher than baseline (Days 30-39) on Days 40 (1.52 ± 0.10 fold increase; P<0.001), 42 (1.66 ± 0.24 fold increase; P=0.02), 43 (2.03 ± 0.29 fold increase; 0.006), and 44 (1.93 ± 0.19 fold increase; P<0.001). Perivulvar temperature, PVT-GT, and PVT-PAT were significantly lower than baseline on Days 41 (1.02 ± 0.37°C, 1.22 ± 0.30°C, and 1.12 ± 0.34°C lower, respectively) and 45 (1.08 ± 0.19°C, 0.51 ± 0.22°C, and 0.76 ± 0.16°C lower, respectively). The measurement which showed the greatest sensitivity to detect subtle changes in body temperature was PVT-GT. Perivulvar temperature relative to gluteal temperature increased 1.96 ± 0.33°C from Day 41 to 43 (P<0.001), corresponding to the period of greatest follicular growth. Both PVT and PVT-GT decreased significantly from Day 43 (i.e., peak follicular activity) to Day 45 (i.e., post-ovulation), which was likely a physiological response to ovulation. In conclusion, both accelerometry and IR thermography shows potential as non-invasive, real-time methods for assessing ovarian activity of cats. However, further investigation into both methods is required.

6.1 Introduction

Accurate monitoring of ovarian cyclicity is important for improving artificial and natural breeding of captive felids (Thongphakdee *et al.*, 2018; Andrews *et al.*, 2019). While a range of methods have been

used to monitor the reproductive state of female felids, many are invasive or not practicable for real-time detection of follicular growth and development (Chapter 1; Andrews *et al.*, 2020). Monitoring the reproductive state of female felids is also often challenging due to high variability in oestrous cycles and, for many species, a lack of overt oestrous behaviours (Foreman, 1997; Wielebnowski and Brown, 1998; Moreira *et al.*, 2001; Brown *et al.*, 2002; Henriksen *et al.*, 2005; Thongphakdee *et al.*, 2018; Andrews *et al.*, 2020).

While there are no clear oestrus-specific behaviours in some felids, oestrus may be associated with subtle increases in the frequencies of locomotion, rubbing, rolling, sniffing, vocalisation, grooming and scent-marking behaviours (Asa *et al.*, 1992; Graham *et al.*, 1995; Wielebnowski and Brown, 1998). These subtle behavioural changes are unlikely to be detected without detailed and labour-intensive behavioural assessment, further complicated by the crepuscular or nocturnal activity of most felids. However, many of the behaviours that felids express more frequently during oestrus appear to correlate with an increase in overall physical activity (OPA; Foreman, 1997; Wielebnowski and Brown, 1998; Moreira *et al.*, 2001; Brown *et al.*, 2002; Andrews *et al.*, 2020). Thus, it may be possible that detecting changes in OPA could improve the accuracy of oestrus detection in felids.

Accelerometry has been validated as an accurate and automated means of assessing OPA of domestic cats (*Felis catus*; Lascelles *et al.*, 2008; Andrews *et al.*, 2015). Accelerometry has been used to detect an increase in OPA associated with oestrus in a range of species including cows (*Bos taurus*; At-Taras and Spahr, 2001; McGowan *et al.*, 2007), mice (*Mus musculus*; Kopp *et al.*, 2006), rats (*Rattus norvegicus*; Gerall *et al.*, 1973), and pigs (*Sus scrofa*; Cornou, 2006). In dairy cattle, accelerometry can accurately detect more than 90% of oestrus events (At-Taras and Spahr, 2001; McGowan *et al.*, 2007). Depending on the magnitude of the change in OPA and the sensitivity of accelerometers to detect such changes, accelerometry may also offer a minimally invasive method for monitoring the ovarian function of in domestic cats.

Another non-invasive method for monitoring ovarian function is infrared (IR) thermography, which has be used to monitor perivulvar temperature (PVT) changes associated with follicular growth and ovulation in cows (Talukder *et al.*, 2014; Radigonda *et al.*, 2017), pigs (Sykes *et al.*, 2012; Simões *et al.*, 2014), horses (*Equus caballus*; Redaelli *et al.*, 2014), and dogs albeit less successfully (Olğaç *et al.*, 2017). Elevated oestradiol concentrations associated with follicular growth have been linked to increased blood flow to the vulva and increased superficial temperature of the perivulvar area (Simões *et al.*, 2014; Talukder *et al.*, 2014). The use of thermographic imaging to monitor the ovarian activity of domestic cats has not yet been investigated.

This study assesses whether accelerometry (monitoring OPA) or IR thermography (monitoring PVT) show potential as non-invasive, real-time methods to monitor ovarian activity in domestic cats.

6.2 Methods

6.2.1 Animals

Twelve healthy, intact female domestic cats aged 2.50 ± 0.22 (1.54 – 3.96) years and weighed 3.12 ± 0.12 kg (mean ± SEM; 2.57 – 3.87 kg) were used for this study. The cats were housed in a purpose-built colony cage at the Centre for Feline Nutrition, Massey University, Palmerston North, New Zealand (175◦3'E, lat. 40°22'S, long.) and fed a complete and balanced (AAFCO, 2020) commercial moist (canned) feline diet (Kraft Heinz Wattie's Ltd., Hastings, New Zealand) with *ad libitum* access to water. The husbandry of the cats complied with the Animal Welfare (Cats) Code of Welfare (Anonymous, 2007) and Massey University Animal Ethics Committee (MUAEC) protocol number 19/10.

6.2.2 Experimental design

The data included in this study were collected during the experiment described in Chapter 4 in which half of the cats (*n*=6) were given 1 mg/kg/day prednisolone (Redipred: Aspen Pharmacare Australia Pty. Ltd., Saint Leonards, NSW, Australia) orally for 45 days. All cats (*n*=12) underwent a standardised ovarian stimulation protocol. Cats were treated with 0.088 mg/kg per day altrenogest (Altreno Oral: Caledonia Holdings Ltd, Auckland, New Zealand), an oral progestin treatment, for 37 days to inhibit follicular growth. On Day 40, the cats were given an intramuscular injection of 75 IU of equine chorionic gonadotrophin (eCG; Novormon: Syntex, Palo Alto, California, USA) to promote follicular growth, with Days 41-44 were defined as a period of 'follicular stimulation'. Eighty hours after the eCG treatment, the cats were treated with an intramuscular injection of 50 IU human chorionic gonadotrophin (hCG; Chorulon: Merck Animal Health, Madison, NJ, USA) to induce ovulation. The cats were anaesthetised and ovariohysterectomised 30-31 hours after the hCG treatment. Ovulation was confirmed by the presence of corpora haemorrhagica or corpora lutea on the ovaries.

Serum oestradiol-17β and progesterone concentrations were analysed to monitor ovarian function at regular intervals throughout the study. Jugular venepuncture was used to collect 2 mL blood samples from each cat on Days 0, 10, 30, and 40 of the trial, as well as 80 hours after eCG treatment, and then 30-31 hours after the hCG treatment (i.e., at the time of ovariohysterectomy). Local anaesthetic (5% lidocaine gel, Emla Cream, AstraZeneca, Cambridge, UK), was applied to the neck of the cats prior to blood collection via jugular venepuncture. Blood samples were centrifuged at 2000 rpm for 10 minutes and serum extracted. Serum samples were stored at -80ºC until assayed. Day 30 was considered as baseline for all assessments as this allowed sufficient time for the regression of any existing luteal tissue (i.e., present at Day 0 and/or 10) and for the progestin treatment to completely suppress follicular growth.

6.2.3 Hormone assessments

6.2.3.1 Serum oestradiol-17β

Serum 17β-oestradiol concentrations were measured using the Cobas® e601 analyser (Hitachi Ltd., Tokyo, Japan) and a commercially available electrochemiluminescence immunoassay, Cobas® Estradiol III (Roche Diagnostics New Zealand, Auckland, New Zealand). Cross-reactivity was <1.0% for all other steroids tested (*n*=35) except for 6-α-hydroxy-oestradiol, which had a cross reactivity of 74.1%. The detection range was 5.0 – 3000.0 pg/mL. Values below the detection threshold were considered 0.0 pg/mL. The mean intra-assay coefficient of variance (CV) was 7.6%.

6.2.3.2 Serum progesterone concentrations

Serum progesterone concentrations were measured using the Cobas® e601 analyser (Hitachi Ltd., Tokyo, Japan) and a commercially available electrochemiluminescence immunoassay, Cobas® Progesterone III (Roche Diagnostics New Zealand, Auckland, New Zealand). Cross-reactivity was <1.0% for all other steroids tested (*n*=27) except for 11-deoxycorticosterone, which had a cross reactivity of 3.9%. The detection range was 0.05 – 60.0 ng/mL. Values below the detection threshold were considered 0.0 ng/mL. The mean intra-assay CV for the quality controls was 2.7%.

6.2.4 Accelerometry

Activity was assessed using Actical® Minimitter accelerometers (MMA; MiniMitter, Bend, Oregon, USA). The MMA measure 28 mm x 27 mm x 10 mm and weigh 17 g. The devices use an omnidirectional accelerometer to detect movement in three planes (craniocaudal, mediolateral and vertical). Actical® accelerometers have been validated for monitoring the activity of domestic cats (Lascelles *et al.*, 2008; Andrews *et al.*, 2015). Actical® accelerometers were attached to the collars of the cats and positioned ventrally (Figure 6.1) one week prior to the beginning of the study, to allow the cats to become accustomed to the devices. Activity data were collected continuously at 1-minute intervals (epochs) throughout the study. No activity data were collected on the last day of the study (Day 45) as the cats were kept in individual cages before and after ovariohysterectomy.

Figure 6.1 Actical® accelerometer fitted to the collar of a cat.

The raw activity data were downloaded from the MMAs using an Actireader® device (MiniMitter, Bend, OR, USA), and activity data (total MMA counts/1 minute) summed to provide a daily (i.e., from 00:00 to 23:59) activity count. Previous research suggests that there is considerable inter-cat variability in average daily MMA counts and the association between MMA activity counts and observed OPA, indicating that care needs to be taken when comparing the activity of cats or assessing the activity data of several cats as a combined data-set (Andrews *et al.*, 2015). To overcome this source of variability, the daily MMA activity counts of each cat were standardised by converting them to a proportion of the average daily activity (i.e., average of the daily activity counts for each cat over the entire study). This was defined as 'proportional daily MMA activity count' and calculated for each cat using the following equation:

$$\text{Proportional daily MMA activity count} = \frac{\text{Daily MMA activity count}}{\text{Average daily MMA activity count}}$$

6.2.5 Infrared thermography

Infrared thermographic images were taken of each cat on Days 30, 36, 37, 38, 39, 40 (eCG treatment day), 41, 42, 43, 44 (hCG treatment), and 45 (ovariohysterectomy). All images were taken using a Flir E60 IR thermographic camera (FLIR Systems, Wilsonville, Oregon, USA) and assessed using the software program Flir Tools (Version 5.13.18031.2002; FLIR Systems, Wilsonville, Oregon, USA). Images were taken 1 m away from the cat in a room with a stable ambient temperature (room thermostat set to 22ºC) after a 15 minute acclimitisation period, although humidity could not be controlled. Two images were taken from each cat on each day (less than 1 minute between images) and results averaged to minimise variability introduced by camera angles or distances on the recorded temperatures. For each image, the average, minimum, and maximum perivulvar temperatures (PVT) were recorded (Figure 6.2). The average left and right gluteal temperatures, as well as the perianal temperature (PAT), were also recorded (Figure 6.2). The PVT relative to the gluteal temperature (PVT-GT) and the PVT relative to the PAT (PVT-PAT) were calculated using the following equations respectively:

1) $\text{PVT} - \text{FT} = \frac{(\text{average PVT} - \text{average left GT}) + (\text{average PVT} - \text{average right GT})}{2}$

2) $\text{PVT} - \text{PAT} = \frac{(\text{average PVT} - \text{average PAT})}{2}$

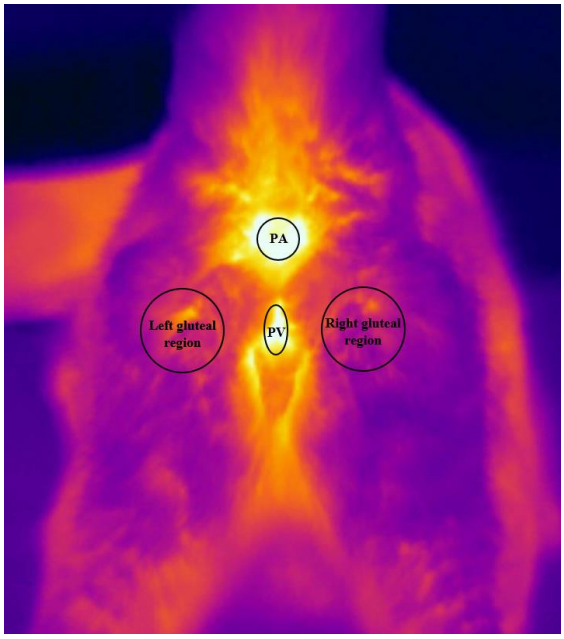

Figure 6.2 Thermographic image of a female cat showing the perivulvar area (PV), perianal area (PA), and the left and right gluteal regions

6.2.6 Statistical analysis

All statistical analyses were conducted using RStudio version 1.0.143 (R Foundation for Statistical Computing, Vienna, Austria) and significance was set as $P<0.05$. A trend or tendency was defined as $P<0.10$ Shapiro-Wilk normality tests were used to assess the normality of the data. Prednisolone treatment administered to six of 12 cats did not significantly affect accelerometer-based activity counts or IR temperature data, thus control and treatment groups as described in Chapter 4 were combined for the purpose of this study. Endocrine data were evaluated for each cat to determine their reproductive state. Chauvenet's criterion was used to remove outlying data points from combined oestradiol data, since one cat had very high baseline and peak oestradiol concentrations. ANOVA and Tukey's post hoc tests were used to assess the endocrine data. The mean daily activity counts on days 40-44 were compared against baseline data (Days 30-39) and were parametric and thus compared using paired T-tests. Proportional daily MMA activity data were also parametric and analysed using paired T-tests. Coefficients of variance were used to examine the amount of variance between the two images captured (less than 1 minute between the images) for each cat daily. PVT, PVT-GT, and PVT-PAT data were parametric and analysed using paired T-tests. Values are presented as mean ± SEM unless otherwise stated.

6.3 Results

6.3.1 Endocrine assessments

The mean oestradiol concentration on Day 0 was 17.8 ± 3.4 pg/ml, which has previously been correlated to a lack of active ovarian follicles (Shille *et al.*, 1979). Oestradiol concentrations spiked

significantly after the eCG treatment on Day 40, being 2.1 ± 0.3 (range: 1.1 - 3.8) fold higher on Day 44 than Day 40 (85.6 ± 14.2 pg/mL vs. 41.8 ± 7.7 pg/mL, respectively; P=0.002; Figure 6.3). Oestradiol concentrations decreased from Day 44 to Day 45 (85.6 ± 14.2 ng/mL vs. 49.5 ± 11.4 ng/mL, respectively; P=0.006), reaching concentration that were comparable to Day 40 (i.e., baseline; P=0.28; Figure 6.3). The stimulation of follicular growth and ovulation was confirmed in all cats by morphological assessment of the ovaries, with an average number of ovulations (i.e., number of corpora haemorrhagica or lutea) of 10.5 ± 1.1 (range: 6 – 22).

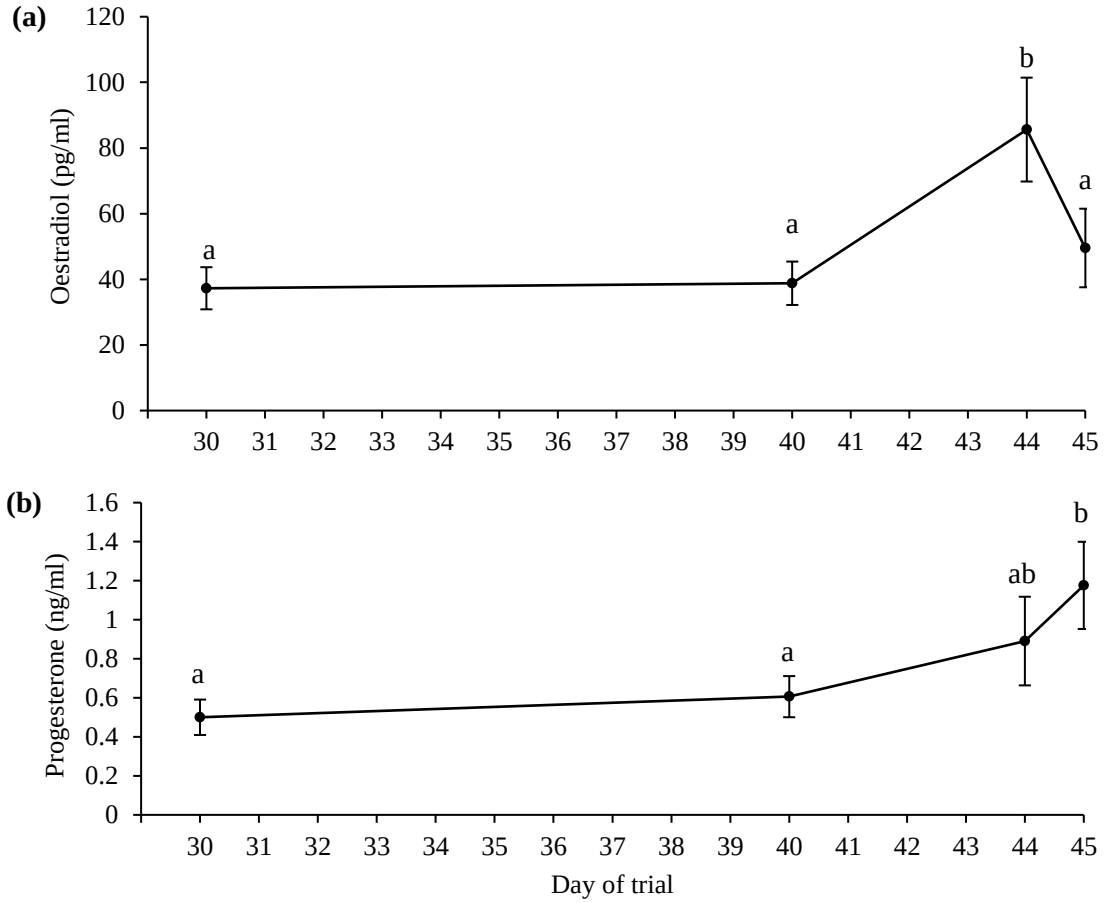

Figure 6.3 Mean (± SEM) serum oestradiol (a) and progesterone (b) concentrations of the cats (n=12) from Day 30-45 of the study. Cats were exposed to an exogenous ovarian control regime consisting of 0.088 mg/kg/day altrenogest (Days 0 – 37), 75 IU equine chorionic gonadotrophin (eCG; Day 40) to stimulate follicular growth, and 50 IU human chorionic gonadotrophin (hCG; Day 44) to induce ovulation. The cats were ovariohysterectomised on Day 45. Day 30 was considered as baseline to allow sufficient time (Days 0-30) for existing (i.e., Day 0) luteal phases to have ceased and the altrenogest treatment to completely suppress follicular growth. Statistical difference (P<0.05) over time is indicated by differing letters.

Three cats had elevated progesterone concentrations at Day 0 (35.3 ± 6.3 ng/ml), two of which also had elevated progesterone (12.6 ng/ml and 36.7 ng/ml) at Day 10, suggesting presence of luteal tissue (i.e. dioestrus). By Day 30, all cats had baseline progesterone concentrations (0.6 ± 0.1 ng/ml), and progesterone concentrations remained low until day 44 (0.9 ± 0.2 ng/ml). However, progesterone

concentrations were higher (2.2 ± 0.6 fold) than baseline on Day 45 (0.5 ± 0.1 ng/mL vs. 1.2 ± 0.2 ng/mL, respectively; P=0.004; Figure 6.3).

6.3.2 Accelerometry

Two MMA malfunctioned; thus, complete activity data were collected from 10 of the 12 cats. On average, there was a 2.2 ± 0.3 fold increase in the daily MMA activity count from baseline (85,477 ± 13,202) to peak (161,895 ± 20,449) levels, with peaks (>1.4 fold increase) in activity being observed on either Day 43 or 44 in all but one cat. However, daily activity counts also differed between cats (14,885 - 307,772 MMA counts/day; P<0.001), with this difference apparent during follicular suppression (14,885 - 197,055 MMA counts/day; P<0.001) and follicular stimulation (22,794 - 307,772 MMA counts/day; P<0.001). The individual variability in daily activity counts was corrected by using proportional daily MMA activity counts. Proportional daily MMA activity counts were higher than baseline (Day 30-39) on Days 40 (1.52 ± 0.10 fold increase; P<0.001), 42 (1.66 ± 0.24 fold increase; P=0.02), 43 (2.03 ± 0.29 fold increase; 0.006), and 44 (1.93 ± 0.19 fold increase; P<0.001; Figure 6.4). Activity levels on day 41 were not different from baseline. Peak activity levels occurred on day 43 (Figure 6.4).

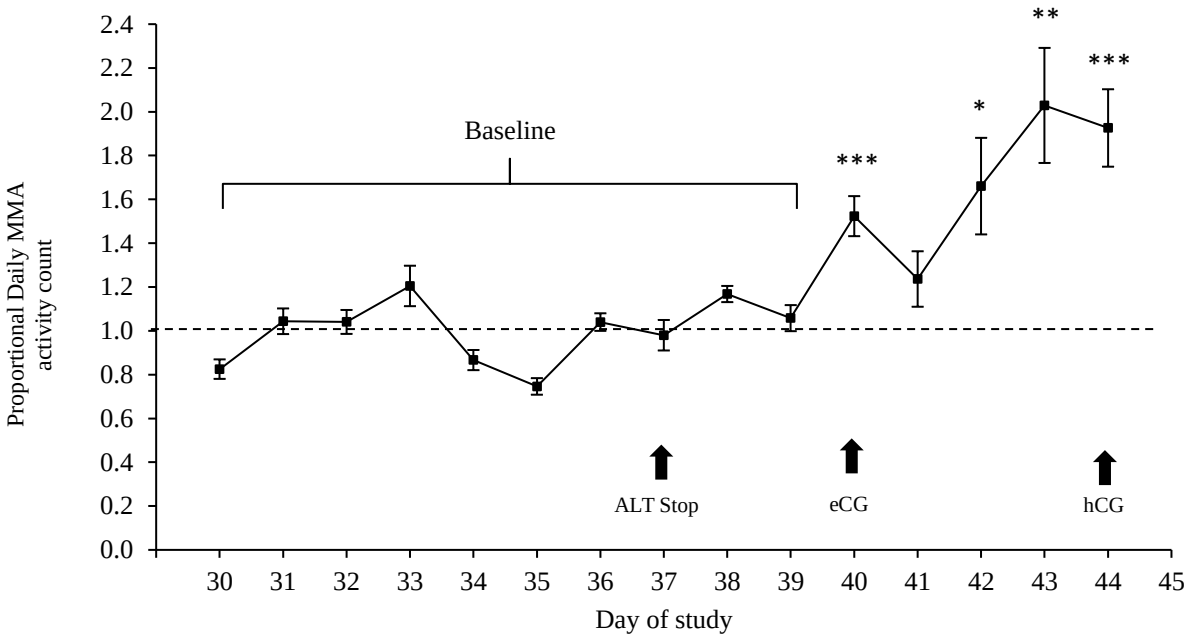

Figure 6.4 Mean proportional daily MMA activity counts of the 10 cats over time. The dotted line represents the overall average proportional daily MMA. Cats were treated with 0.088 mg/kg/day altrenogest (ALT) orally from Days 0 – 37. On day 40, the cats were given 75 IU equine chorionic gonadotrophin (eCG) to induce follicular growth, followed by 50 IU human chorionic gonadotrophin (hCG) to induce ovulation 80 hours later. The cats were ovariohysterectomised 24 hours after the hCG treatment (i.e., Day 45). The proportional mean MMA activity counts on Days 40, 41, 42, 43, and 44 were each compared against baseline levels (Days 30-39; indicated by the dotted line) using paired T-tests. *P<0.05, **P<0.01, ***P<0.001.

6.3.3 Infrared thermography

There was little variation in the PVT and PVT-GT recordings between the two images per cat each day, with mean CV of 0.88 ± 0.08% and 3.02 ± 0.21%, respectively. However, PVT-PAT differed considerably between two daily images per cat (CV of 34.75 ± 5.60%).

The mean baseline (Days 30-39) PVT was 36.59 ± 0.45°C. Mean PVT were lower than baseline on Days 40 (0.75 ± 0.21°C lower; P=0.006), 41 (1.02 ± 0.37°C lower; P=0.02), 44 (0.65 ± 0.23°C lower; P=0.03), and 45 (1.08 ± 0.19°C lower; P<0.001; Figure 6.5). However, PVT was similar to baseline on Days 42 (P=0.12) and 43 (P=0.44; Figure 6.5). While PVT appeared to increase from Days 41 to 43 (Figure 6.5), this was not significant (P=0.15). Interestingly, PVT decreased (0.89 ± 0.28°C) from Days 43 (peak follicular stimulation) to 45 (P=0.008; Figure 6.5), which corresponded with the post-ovulatory period.

Perivulvar temperature relative to mean gluteal temperature followed a similar pattern to PVT (Figure 6.5). The mean baseline PVT-GT was 12.24 ± 0.56°C. PVT-GT differed from baseline on Days 41 (1.22 ± 0.30°C lower; P=0.002), 43 (0.74 ± 0.29°C; P=0.03), and 45 (0.51 ± 0.22°C lower; P<0.001). There was a 0.97 ± 0.38°C decrease in PVT-GT from Days 40 to 41 (P=0.03; Figure 6.5), and a significant increase from Days 41 to 43 (1.96 ± 0.33°C). As with PVT, PVT-GT decreased 1.24 ± 0.41°C from Days 43 to 45 (P=0.01).

The stimulation of follicular growth and ovulation with gonadotrophins appeared to have a similar effect on PVT-PAT. The mean baseline PVT-PAT was -0.78 ± 0.51°C. As with PVT and PVT-GT, PVT-PAT decreased after the stimulation of follicular growth and ovulation with gonadotrophins (Figure 6.5). PVT-PAT was lower than baseline on Days 40 (0.66 ± 0.17°C lower; P=0.002), 41 (1.12 ± 0.34°C lower; P=0.007), and 45 (0.76 ± 0.16°C lower; P<0.001; Figure 6.5). PVT-PAT appeared to increase from Days 41 to 43, although this was not significant. There was a decrease in PVT-PAT from Days 43 to 45 (Figure 6.5), but unlike for PVT and PVT-GT, this difference was not significant.

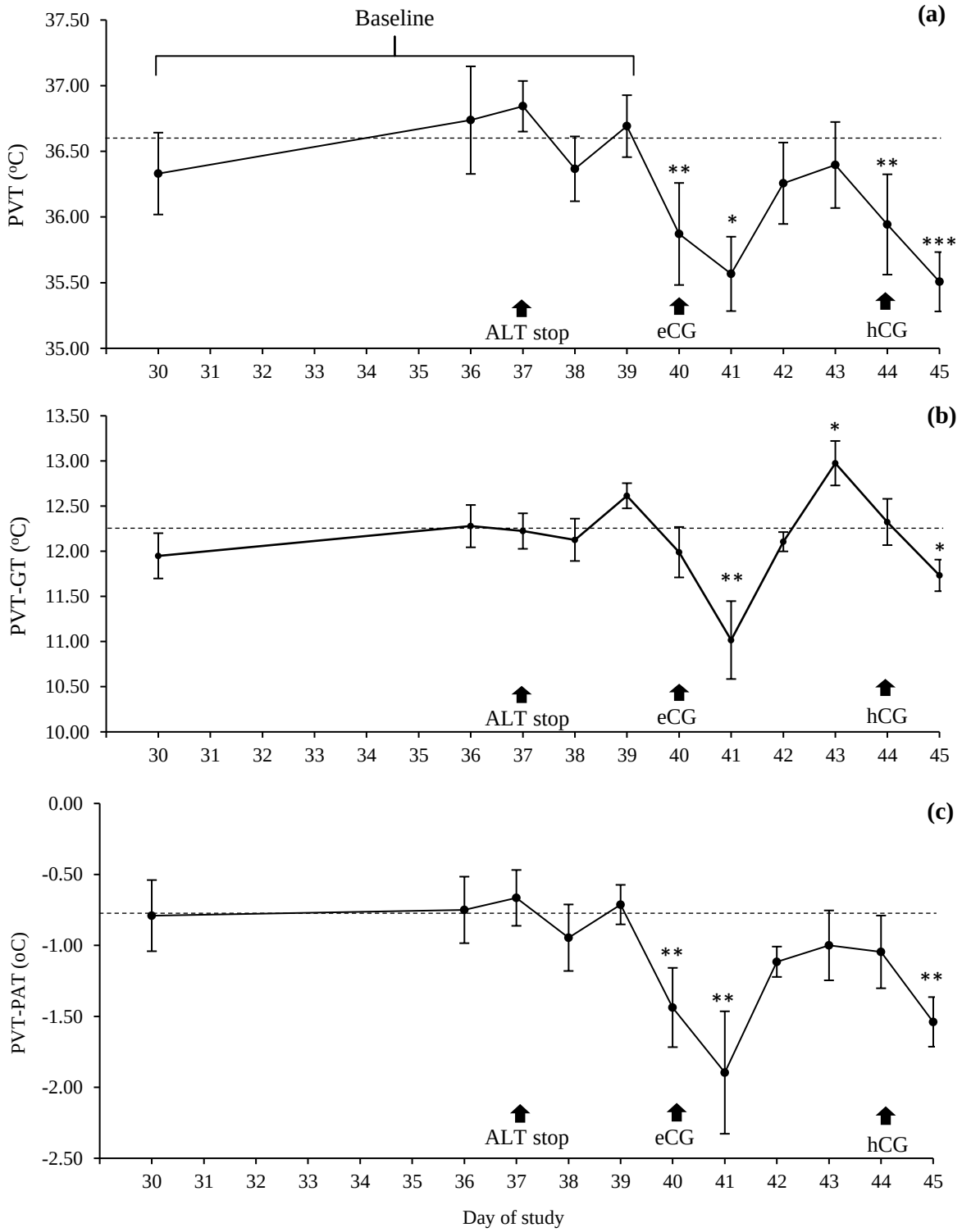

Figure 6.5 (a) Perivulvar temperature (PVT), (b) PVT relative to gluteal temperature (PVT-GT), and (c) PVT relative to perianal temperature (PVT-PAT) of the cats (n=12) over time. Cats were treated with 0.088 mg/kg/day altrenogest (ALT) orally from Days 0 – 37. Complete ovarian suppression was achieved in all cats by Day 30. On Day 40, the cats were given 75 IU equine chorionic gonadotrophin (eCG) to induce follicular growth, followed by 50 IU human chorionic gonadotrophin (hCG) to induce ovulation 80 hours later. The mean PVT, PVT-GT, and PVT-PAT on Days 40, 41, 42, 43, and 44 were each compared against baseline levels (Days 30-39; indicated by the dotted line) using paired T-tests. *P<0.05, **P<0.01, ***P<0.001.

6.4 Discussion

This study investigated whether accelerometry and IR thermography could be used as tools for monitoring the ovarian function of domestic cats. Accelerometry data indicated that the OPA of cats increased significantly following the induction of follicular growth with eCG. In this study, a peak in activity following the stimulation of follicular growth with eCG was observed in 90% of the cats, suggesting that accelerometry could be used as a tool to aid in the detection of oestrus in this species. Similarly, IR thermography was successful in identifying significant differences in the PVT and PVT-GT of cats based on whether they were undergoing ovarian suppression (baseline), peak follicular activity (Day 43), and post-ovulation (Day 45).

The present study demonstrates that accelerometry could be used to detect the behavioural changes (increased OPA) associated with follicular growth without the need for extensive observation monitoring. The OPA of the cats increased progressively after the induction of follicular growth, reaching peak activity levels three days after the eCG treatment. It has been well documented that eCG treatment in domestic cats triggers the development of pre-ovulatory follicles within two to three days, hence hCG is given to induce ovulation ~80 hours (i.e., 3.33 days) after the eCG treatment (Swanson *et al.*, 1997; Stewart *et al.*, 2012). Oestradiol concentrations increase gradually with follicular growth and peak in the presence of pre-ovulatory follicles (Griffin, 2001; Chatdarong, 2003; Bristol-Gould and Woodruff, 2006; Malandain *et al.*, 2011). Furthermore, the behavioural changes associated with oestrus in domestic cats have been directly linked to elevated oestradiol concentrations (Michael and Scott, 1964; Shille *et al.*, 1979; Wildt *et al.*, 1981a; Schmidt *et al.*, 1983). In this study, the increases in the OPA of the cats in this study following the eCG treatment paralleled that of serum oestradiol concentrations.

Induction of follicular growth using eCG is known to induce the development of more pre-ovulatory follicles than in natural follicular waves (Goodrowe *et al.*, 1988b; Donoghue *et al.*, 1993; Roth *et al.*, 1997). Whether the OPA levels of domestic cats also increases during periods of natural oestrus remains to be confirmed. The number of ovulations observed here (10.5 ± 1.1 ovulations per cat, range: 6 - 22) is comparable to other studies that have induced oestrus and ovulation using exogenous gonadotrophins (9-15 ovulations per cat), but was higher than reported for natural oestrus and ovulation (~5.0 ovulations per cat; Wildt *et al.*, 1981a; Goodrowe *et al.*, 1988b; Donoghue *et al.*, 1993; Swanson *et al.*, 1997; Stewart *et al.*, 2012). It would have been interesting to investigate activity changes in response to ovulation and monitor physical activity at the time of ovulation, but this could not be accurately assessed as the cats used here were housed in individual cages (restricted areas) the night prior to ovariohysterectomy. Nonetheless, the findings of the present study justify further research into the use of accelerometry (i.e., OPA monitoring) for detecting oestrus in domestic cats, and more importantly, non-domestic felids that typically lack overt behavioural indicators of oestrus.

Infrared thermography was successful in identifying the transitions between different ovarian statuses within cats, from ovarian inactivity (i.e., baseline or Days 30-39), to peak ovarian follicle activity and oestradiol concentrations (Days 43 and 44), and lastly to the post-ovulatory stage (Day 45; Figure 6.5). The decrease in temperature registered by IR thermography on day 41 was consistent, although the physiological cause and biological significance of this remains unclear. Finally, from all of the parameters that were derived from IR thermography (PVT, PVT-GT, and PVT-PAT), PVT-GT provided the best sensitivity to detect subtle changes in body temperature. In addition, there was little variation in PVT and PVT-GT determined from the two images captured (less than 1 minute between the images) for each cat daily (0.88% and 3.02% variation, respectively), suggesting that there is no need for capturing multiple images of each animal at any given time point. However, there was some inter-individual variation in the PVT, PVT-GT, and PVT-PAT changes associated with the gonadotrophin treatments, emphasising the requirement for baseline comparisons for each animal.

Many studies that have used IR thermography to monitor reproductive state have reported or shown a high degree of variability in the temperatures obtained (Sykes *et al.*, 2012; Simões *et al.*, 2014; Talukder *et al.*, 2014; Olğaç *et al.*, 2017; Radigonda *et al.*, 2017). Some authors have attributed this variability to a range of external variables (e.g., ambient temperature, humidity, air movement, focal distance, physical activity of subject, and fur density and coverage) that have been shown to affect body temperature and IR thermography data (Kastberger and Stachl, 2003; Cilulko *et al.*, 2013; Rekant *et al.*, 2016; Olğaç *et al.*, 2017). In the present study, all thermographic images were taken indoors under stable environmental conditions, so the variability observed in cats was unlikely due to environmental factors. The amount of physical activity of the subject prior to taking a thermographic image has been found to cause variation in the recorded temperatures (Simões *et al.*, 2014; Silva *et al.*, 2017). In the present study, there was an increase in physical activity following the eCG treatment, with both follicular growth and OPA levels increasing progressively from Day 41 to Day 43. It is possible that the increase in PVT from Days 41 to 43 was due, in part to the increase in OPA. However, the use of relative temperature measures (PVT-GT and PVT-PAT) should have accounted for temperature changes that may originate from different activity levels. Additionally, given that the increases in activity were associated with follicular growth, activity-induced changes in PVT in the context of monitoring ovarian activity are probably of minimal significance. The results presented herein show that PVT-GT, and to a lesser extent PVT-PAT, increased from Days 41 to 43 as did PVT, suggesting that this increase represents a true physiological response (i.e., due to increases in follicular activity and/or oestradiol concentrations) and not a consequence of increased OPA.

The experimental design of this study highlighted important considerations for future studies. First, IR images were only collected once per day. More frequent collection of images might enable more accurate identification of the timing of peak follicular growth and ovulation. Second, IR images were taken at a fixed time each day rather than relative to when the eCG and hCG treatments were

administered, so the timing of the IR images varied from nine to 15 hours after eCG treatment. This variation almost certainly caused inconsistencies in the stage of follicular growth or ovulation as determined from the infrared images. Nevertheless, the results of this study are encouraging and provide impetus for further investigation into the use of IR thermography for monitoring ovarian function in cats.

6.5 Conclusion

In conclusion, this study shows the potential for accelerometry and IR thermography to be used as a minimally-invasive method for monitoring the ovarian activity of cats, and perhaps other felid species. The OPA increased after the induction of follicular development with eCG, but the Actical® activity counts differed considerably among cats. In terms of detecting follicular growth or ovulation in cats, this means that comparative baseline (i.e., no follicular activity) activity levels are required for each animal. Future research into whether OPA also increases during periods of natural oestrus would be worthwhile, as the induction of follicular growth with eCG results in a stronger ovarian response than observed during natural oestrus. Infrared thermography also identified PVT changes driven by follicular development and ovulation, with PVT-GT providing the most sensitive method for detecting the subtle PVT changes associated with gonadotrophin-induced follicular growth and ovulation. Thus, the findings of the present study indicated that both accelerometry) and IR thermography show promising for monitoring the ovarian activity of cats.

Chapter 7

General discussion and future research

Chapter 7: General discussion and future research

7.1 General discussion

The major threats (e.g., habitat loss/fragmentation, reductions in prey numbers, and poaching) faced by many felids have made *in situ* conservation programs extremely difficult (Nowell and Jackson, 1996; Inskip and Zimmermann, 2009; Palazy *et al.*, 2011; Poessel *et al.*, 2014; Wolf and Ripple, 2016; 2017). Consequently, captive breeding programs have become pivotal to conservation strategies for non-domestic felids. While natural breeding is optimal for sustaining captive felid populations, it is often challenging due to the need for animal transportation, intra-specific aggression, mate incompatibility, and difficulties in oestrus detection (Foreman, 1997; Wielebnowski and Brown, 1998; Moreira *et al.*, 2001; Brown *et al.*, 2002; Wielebnowski *et al.*, 2002a; Henriksen *et al.*, 2005; DeCaluwe *et al.*, 2013; Thongphakdee *et al.*, 2018; Andrews *et al.*, 2019; Andrews *et al.*, 2020; Thongphakdee *et al.*, 2020). Assisted reproductive technologies (ART) provide methods for transferring genes between *ex situ* organisations, and perhaps from *ex situ* to *in situ* populations, but have generally had low success rates in felids (Howard and Wildt, 2009; Thongphakdee *et al.*, 2020). This thesis aimed to identify and examine factors that influence the reproductive performance of felids in captivity. This first required a detailed understanding of the general reductive biology of felids.

Chapter 2 provided the first comprehensive systematic review of the literature available on the reproductive biology of all extant felid species (Andrews *et al.*, 2019; Andrews *et al.*, 2020). The review highlighted that teratospermia appeared to be the most significant factor affecting the fertility of male felids (Howard *et al.*, 1990; Long *et al.*, 1996; Pukazhenthi *et al.*, 2006b; Andrews *et al.*, 2019). In females, highly variable oestrous cycles and difficulties in detecting oestrus complicate captive breeding programs (Foreman, 1997; Wielebnowski and Brown, 1998; Brown *et al.*, 2002; Henriksen *et al.*, 2005; Moreira *et al.*, 2007; Thongphakdee *et al.*, 2018; Andrews *et al.*, 2019; Andrews *et al.*, 2020).

Captivity has been found to affect both the testicular and ovarian function of felids (Jurke *et al.*, 1997; Wielebnowski *et al.*, 2002b; Moreira *et al.*, 2007; Koester *et al.*, 2015; Andrews *et al.*, 2019). However, it is difficult to elucidate whether differences in the ejaculate characteristics or ovarian cyclicity between captive and wild felids are due to captivity-related stress (i.e., elevated glucocorticoid (GC) concentrations) or other factors associated with captivity (e.g., diet). While a number of captive felid species have been found to exhibit higher (~2.5 fold) basal faecal glucocorticoid (GC) concentrations than their wild conspecifics (Wielebnowski *et al.*, 2002a; Wielebnowski *et al.*, 2002b; Terio *et al.*, 2004; Fanson *et al.*, 2010; Fanson *et al.*, 2012), there is a lack of evidence for a direct effect of stress (i.e., increased hypothalamic-pituitary-adrenal activity) on

felid reproduction (Andrews *et al.*, 2019). Furthermore, to the best of my knowledge, no study to date has examined the effects of elevated GC on the follicular growth, ovulation rate, or oocyte quality of felids following exogenous stimulation of ovarian activity.

Chapters 3 and 4 examined the effects of a simulated endocrine stress response (i.e., administration of a physiologically relevant dose of prednisolone) on the testicular and ovarian function of domestic cats (*Felis catus*), respectively. While prednisolone was administered to the treatment cats at 1 mg/kg/day, prednisolone was not detectable in the serum any of the animals (treatment or control). This was likely because blood samples were taken ~22-24 hours after the prednisolone treatment was administered. Initial investigations suggested that prednisolone was an intermediate-acting GC, with a half-life of 12-36 hours reported in companion animals (Behrend and Kemppainen, 1997; Lowe *et al*., 2008a). However, further retrospective investigation into the half-life of prednisolone in humans indicated that it has a much shorter life of 2-3 hour (Rose *et al*., 1981). This shorter half-life would likely have meant that complete drug clearance occurred within 22 hours. Despite no detectable differences in the serum prednisolone concentrations between the control and treatment cats, physiological effects of the prednisolone treatment on the testicular and ovarian function of the cats were apparent.

Higher proportions of morphological abnormal sperm were observed in the epididymides of prednisolone-treated cats than control cats (Chapter 3). The mechanism of this was unclear as both primary (associated with spermatogenesis) and secondary (associated with maturation) abnormalities were more prevalent in cats given prednisolone (Pukazhenthi *et al.*, 2001; Pukazhenthi *et al.*, 2006b; Crosier *et al.*, 2009; Müller *et al.*, 2012). Reductions in Sertoli cell number (via increased Sertoli call apoptosis) and an increased Sertoli cell load (i.e., germ cells per Sertoli cell) may have contributed towards the higher proportion of abnormal sperm in prednisolone-treated cats (Mruk and Cheng, 2004; Wang *et al.*, 2006; Johnson *et al.*, 2008). Regardless of the aetiology, this would likely have a detrimental effect on fertility, since morphologically abnormal sperm are rarely involved in the fertilisation process and can even disrupt the functioning of structurally normal sperm (Howard *et al.*, 1990; Long *et al.*, 1996; Pukazhenthi *et al.*, 1996; Pukazhenthl *et al.*, 1998; Terrell *et al.*, 2010).

The effects of the prednisolone treatment on ovarian function and oocyte quality of cats following exogenous simulation of follicular growth and ovulation appeared to be minimal (Chapter 4). Oocytes collected from prednisolone-treated cats had less uniform ooplasms and poorer zona pellucida quality than those collected from control cats (Chapter 4). However, it is unclear whether the reduced ooplasm and zona pellucida scores of prednisolone-treated cats would correspond to a reduction in fertility, as the fertilisation capability of oocytes was not assessed.

The results of Chapters 3 and 4 suggest that a simulated endocrine stress response has a greater effect on the testicular function of toms than the ovarian function of queens, at least when ovarian function

is controlled using exogenous gonadotrophins. This suggests that careful management (low stress) of male cats should be prioritised, especially for the 50 days (the duration of spermatogenic cycle is approximately 47 days) prior to breeding or ejaculate collection for ART, since follicular growth and ovulation is almost always stimulated with exogenous gonadotrophins for ART.

The hyperstimulation of follicular growth and ovulation with pharmacological doses of exogenous gonadotrophins likely compensated for any effects of the prednisolone treatment on the ovarian function of the cats (Goodrowe *et al.*, 1988b; Donoghue *et al.*, 1993). The simulation of follicular growth and ovulation in cats using exogenous gonadotrophins has been shown to result in a higher number of ovulations per cat than natural oestrus and ovulation (either induced or from natural mating; Wildt *et al.*, 1981a; Goodrowe *et al.*, 1988b; Donoghue *et al.*, 1993; Swanson *et al.*, 1997; Stewart *et al.*, 2012). Future research should focus on the effects of GC administration on the natural oestrous cycles of cats, as elevated GC concentrations have been associated with ovarian quiescence in cheetah (*Acinonyx jubatus*; Jurke *et al.*, 1997), margay (*Leopardus weidii*), and trigrina (*Leopardis tigrinus*; Moreira *et al.*, 2007). However, this requires an accurate, reliable, and minimally invasive (i.e., low stress) method for monitoring ovarian cycles of domestic cats. Thus, Chapter 6 evaluated whether accelerometry (activity monitoring) and/or infrared (IR) thermography (perivulvar temperature (PVT) monitoring) could be used to monitor the ovarian function of domestic cats, as a model for endangered felids.

Actical® (MiniMitter, Bend, OR, USA) accelerometers have been validated for monitoring overall physical activity (OPA) of domestic cats (Andrews *et al.*, 2015). However, these devices are monoaxial and can only monitor OPA (Lascelles *et al.*, 2008; Andrews *et al.*, 2015). Chapter 5 therefore aimed to validate and develop Heyrex® activity monitors, as these triaxial accelerometers were capable of monitoring both the activity and behaviour (e.g., sleeping, alert, scratching, walking, and running) of cats. Furthermore, Heyrex® activity monitors also have a long battery-life (~2 years) and wirelessly upload data onto a server that can be accessed anywhere in the world, thus enabling real-time monitoring.

The results of Chapter 5 indicated that the Heyrex® devices provided an accurate representation of OPA of cats; moreover, the regression lines between observed and Heyrex® activity counts were more similar among cats than for previously tested accelerometers (Andrews *et al.*, 2015). However, refinement of the behaviour algorithms is required, with the devices frequently miscategorising scratching and walking behaviour. In addition, it was concluded that grooming behaviour needs to be incorporated into the behavioural algorithms, as cats spent a considerable amount time grooming and this affected the categorisation of behaviours by the Heyrex® devices. Unfortunately, algorithm and device modification for cats was not possible as Heyrex® Ltd. closed down; moreover, the online

servers were suspended, which meant that activity data could no longer be collected from the Heyrex® devices. Consequently, Actical® accelerometers were used in Chapter 6.

Chapter 6 demonstrated potential for both accelerometry and IR thermography to be used to monitor the ovarian function of cats. The OPA of the cats peaked three days after the equine chorionic gonadotrophin (eCG) treatment, which corresponded to peak follicular activity and oestradiol concentrations. Past literature has also shown that pre-ovulatory follicles are present within two to three days of stimulating follicular growth with exogenous gonadotrophins (Swanson *et al.*, 1997; Stewart *et al.*, 2012). Furthermore, there is direct evidence in cats that behavioural changes during oestrus are directly due to the elevated oestradiol concentrations associated with follicular growth (Michael and Scott, 1964; Shille *et al.*, 1979; Wildt *et al.*, 1981a; Schmidt *et al.*, 1983). Thus, elevated oestradiol concentrations are the most likely cause for the increased OPA of cats following the eCG treatment.

In cows (*Bos taurus*) and pigs (*Sus scrofa*), elevated oestradiol concentrations associated with follicular growth have also been linked to increased blood flow to the perivulvar area and, in turn, increased superficial temperature of the perivulvar area (Simões *et al.*, 2014; Talukder *et al.*, 2014). In Chapter 6, the PVT (determined using IR thermography) of cats changed following exogenous gonadotrophin stimulation of follicular growth and ovulation. Perivulvar temperature relative to gluteal temperature (PVT-GT) had the greatest sensitivity for detecting subtle changes in PVT. There was a decrease in PVT-GT within one day of eCG-induced follicular growth, which likely corresponded to early follicular growth (Goodrowe and Wildt, 1987; Swanson *et al.*, 1997; Stewart *et al.*, 2012). Interestingly, PVT-GT increased as follicles developed and reached peak levels three days after the eCG treatment, which was also when follicular activity and OPA peaked. Both PVT and PVT-GT decreased following induction of ovulation with human chorionic gonadotrophin (hCG). This suggests that PVT monitoring has potential to be used to identify ovulation in cats, as in some non-felid species (Sykes *et al.*, 2012; Redaelli *et al.*, 2014; Simões *et al.*, 2014; Talukder *et al.*, 2014; Radigonda *et al.*, 2017).

Considerable inter-individual variation existed in the OPA (accelerometer) and PVT (IR thermography) data. A high degree of inter-cat variation in accelerometry data has been reported previously (Andrews *et al.*, 2015). In terms of detecting follicular growth or ovulation in cats, this means that an activity profile is required for each animal. This is also applicable for PVT data, as numerous external variables (e.g., ambient temperature, humidity, air movement, focal distance, physical activity of subject, and fur density) can affect body temperatures and, thus, IR thermography data (Kastberger and Stachl, 2003; Cilulko *et al.*, 2013; Rekant *et al.*, 2016). Nevertheless, the results of Chapter 6 are encouraging and provide impetus for further investigation into the use of both

accelerometry and IR thermography for monitoring ovarian function of cats and, perhaps, non-domestic felids.

7.2 Future directions

- In Chapter 3, secondary sperm abnormalities (particular proximal cytoplasmic droplets) were higher in prednisolone-treated cats than control cats. Secondary abnormalities occur during maturation. Testosterone has an important role in regulating epididymal sperm transport and maturation, with low testosterone concentrations being linked to an increase in the proportion of morphologically abnormal sperm in mice (Meistrich *et al.*, 1975). Serum testosterone concentrations were comparable between prednisolone-treated and control cats. However, androgen binding protein also has an important role on regulating testicular androgen concentrations and concentrating testosterone in the epididymis (Munell *et al.*, 2002). Thus, it would be worth investigating whether androgen binding protein concentrations differed in the testes of the treatment and control cats in Chapter 3.

- Glucocorticoids have been found to decrease luteinising hormone (LH) receptor expression in both testicular and ovarian tissue *in vitro* (Bambino & Hsueh, 1981; Kawate et al., 1993). Glucocorticoids also dose-dependently inhibit the production of oestradiol from follicle-stimulating hormone (FSH)-treated and androstenedione-treated bovine and rat granulosa cells *in vitro*, with research suggesting that GC act to disrupt the FSH-induced increases in P450 aromatase activity (Hsueh & Erickson, 1978; Kawate et al., 1993). It would be valuable to examine whether the prednisolone treatment altered LH receptor and FSH receptor expression in the testicular and ovarian tissues collected in Chapters 3 and 4, respectively. Determining whether gonadotrophin receptor expression differs between treatment and control cats may shed light on the physiological pathways by which the GC treatment affected the testicular and ovarian functions.

- In Chapter 4**,** it was likely that the hyper-stimulation of follicular growth and ovulation with exogenous gonadotrophins mitigated the effects of GC on ovarian functions. While elevated GC concentrations associated with captivity have been associated with ovarian quiescence in felids (Jurke *et al.*, 1997; Moreira *et al.*, 2007), no direct physiological link has been reported between elevated GC and reduced oestrous cyclicity in felids. Therefore, a study that evaluated the effect of GC treatments on natural ovarian cyclicity would be valuable.

- This thesis found that GC treatments resulted changes morphological characteristics of both the sperm and oocytes of cats. However, whether these morpholigcal changes affect the fertility of cats remains to be determined. In the future, it would be worthwhile examining

whether GC treatments affect factors such as functional indicators of fertilisastion ability such as sperm binding to the ZP, *in vitro* fertilisation rats, and embryo formation.

- Chapter 6 also showed that cats exhibit an increase in OPA following induction of follicular growth with eCG, indicating that that accelerometry could be used as a non-invasive (low stress) method for monitoring ovarian activity. The ability to accurately monitoring the reproductive state of female cats would likely facilitate natural breeding of felids in captivity. Thus, future research into whether the OPA also increases during periods of natural (i.e., unstimulated) oestrus would be valuable. In Chapter 6, it was not possible to study the effects of ovulation on OPA, because the cats were kept in smaller individual cages after the hCG treatment in accordance with pre-anaesthesia protocols. The effects of ovulation on OPA is worth investigating, especially given the potential benefits for ART of being able to detect ovulation in other felids.
- Chapter 6 indicated that IR thermography can possibly be used as a non-invasive method or monitoring the ovarian activity of cats, with PVT changing through periods of follicular growth and ovulation. However, a limitation of the research in Chapter 6 was that IR images were taken at a fixed time each day rather than relative to when the eCG and hCG treatments were administered. While this was a consequence of the research in Chapter 4 and 6 being conducted simultaneously in the same cohort of cats, it almost certainly resulted in the images being taken at slightly different stage of follicular development. Future research should consider synchronising the time of IR imaging with exogenous gonadotrophin treatments, with IR images being taken more frequently (e.g., three times daily) than in Chapter 6.
- The results of this thesis indicate that GC have adverse effects on the testicular and ovarian function of domestic cats. There is an urgent need to further investigate the effects of captivity-related stress on non-domestic felids, particularly in male felids. Furthermore, the non-invasive approaches used for monitoring ovarian activity in this thesis are highly applicable for the management and breeding of non-domestic felids in captivity.

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Appendix 1

Publication

Andrews C.J., Thomas D.G., Yapura J., Potter M.A. (2019) Reproductive biology of the 38 extant felid species: A review. *Mammal Review* 49, 16-30.

Appendix 1: Publication: Andrews C.J., Thomas D.G., Yapura J., Potter M.A. (2019) Reproductive biology of the 38 extant felid species: A review. *Mammal Review* 49, 16-30.

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Christopher Jon Andrews
Name/title of Primary Supervisor:	Assoc. Prof. David Thomas
Name of Research Output and full reference:	
Andrews C.J., Thomas D.G., Yapura J., Potter M.A. (2019) Reproductive biology of the 38 extant felid species: A review. Mammal Review 49, 16-30.	
In which Chapter is the Manuscript /Published work:	Chapter 2
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	60%
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
Chris Andrews collated the literature, analysed the data, and wrote the initial draft. The author group then completed the editing phase together.	
For manuscripts intended for publication please indicate target journal:	
This article was published in the peer-reviewed journal, Mammal Review.	
Candidate's Signature:	Christopher J Andrews Digitally signed by Christopher J Andrews Date: 2021.02.18 21:08:03 +13'00'
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(This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis)

Appendix 1b: Andrews *et al.* (2019) Supplement 2 (S2) – Data on the oestrous cycles of felids

A summary of the existing literature (84 publications) on the female reproductive biology of the 38 extant felid species. The felid lineages and associated species are based on Johnson et al. (2006). All values are presented as weighted mean$^{\mu}$ (range; n = number of females, n_E = number of events (oestrous cycle length, oestrus, interoestrus, non-pregnant luteal phases (often referred to as pseudopregnancy), and pregnancy)), or as otherwise stated. Many of the values for the duration of interoestrus were calculated from the mean durations and ranges of oestrus and the full oestrus cycle. Calculated values are represented by ‡. The type of data provided by each reference is indicated by the following key: behavioural (B), endocrine (E), histological (H), laparoscopic (L), observational (O), review article (R), transabdominal ultrasonography (U) or vaginal cytology (V). Values reported in the review article by Rodrigues da Paz (2012), indicated in the table as “RdP 2012”, were excluded from all calculations as sample sizes were unknown. Similarly, values marked with an * are from Hunter and Barrett (2011) and have been used when primarily literature is lacking or limited; these values have also been excluded from all analyses. Values marked with † refers to the persistent nature of corporal lutea (CL) in the *Lynx* spp., with luteal phases in these species being found to persist for as long two years (Jewgenow et al. 2014, Painer et al. 2014). Progesterone concentrations remain elevated throughout this time, although remaining CL are functionally supressed during the next breeding season to enable follicular development to occur and thus the onset of oestrus (Jewgenow et al. 2014, Painer et al. 2014). Other abbreviations: northern hemisphere (NH), southern hemisphere (SH), non-seasonal (NS), inter-quartile range (IQ).

Lineage	Species	Age of female sexual maturity (Months)	BWT of adult female (kg)	Cyclicity (poly/mono-oestric)	Breeding season	Length of anovulatory oestrous cycle (Days)	Duration of oestrus (Days)	Interoestrus interval (Days)	Occurrence of spontaneous ovulation	Dioestrus: Non-pregnant luteal phase (Days)	Dioestrus: Pregnant luteal phase/Gestation (Days)	Litter size (range)	References
Domestic Cat	**Domestic cat** (*Felis catus*)	4.0 – 12.0	2.0 – 5.0	Poly	Feb - Sept (NH)	18.9$^{\mu}$ (1.5 – 68.5) (*n = 38, n_E = 213*)	7.3$^{\mu}$ (0.5 – 118) (*n = 129, n_E = 454*)	9.9$^{\mu}$ (1.5 – 64.5) (*n = 45, n_E = 173*)	Regular	40.3$^{\mu}$ (26 – 55) (*n = 28, n_E = 61*)	66.9 (62 – 71) (*n = 14, n_E = 15*)	1 – 5 (*n = 15*)	Paape et al. 1975[E], Verhage et al. 1976[E], Shille et al., 1979[B,E,V], Shille & Stabenfeldt 1979[E], Wildt et al. 1981[B,E], Tsutsui & Stabenfeldt 1992, Root et al. 1994[E], Chatdarong et al. 2006[E,V], da Silva et al. 2006[B], Stewart et al. 2010[E]
	European/Scottish wildcat (*Felis silvestris*)	9.0 – 12.0*	2.0 – 5.8*	Poly	Oct - Feb	-	-	-	-	-	56.0 – 68.0*	4.3 (3 – 6) (*n = 9*)	Matthews 1941[H], Daniels et al. 2002[H]
	African wildcat (*Felis libyca*)	9.0 – 12.0*	2.0 – 7.7*	Poly	-	-	-	-	-	-	67.0 (*n = 1, n_E = 1*)	1 – 6*	Dehnhard et al. 2012[E]
	Chinese mountain cat (*Felis bieti*)	-	6.5 – 9*	-	-	-	-	-	-	-	-	-	
	Desert/Sand cat (*Felis margarita*)	9.0 – 14.0*	1.4 – 3.1*	Poly	NS	11.1 (5 – 28) (*n = 6, n_E = 109*)	2.9 (1 – 11) (*n = 6, n_E = 109*)	9.0 (4 – 27) ‡	Rare	-	64.2 (61 – 67) (*n = 5, n_E = 5*)	2 – 5, usually 2*	Mellen 1993[B,O], Herrick et al. 2010[E], Dehnhard et al. 2012[E]
	Black-footed cat (*Felis nigripes*)	~7.0*	1 – 1.6*	Poly	NS	11.9 (5 – 29) (*n = 7, n_E = 98*)	2.2 (1 – 9) (*n =7, n_E = 98*)	9.9 (4 – 28) ‡	Regular	13.2±1.1 (*n = 5, n_E = 26*)	61.0 (60 – 62) (*n = 2, n_E = 2*)	Usually 2*	Herrick et al. 2010[E]
	Jungle cat (*Felis chaus*)	~11.0*	2.6 – 9*	Poly	-	-	-	-	-	-	65.3 (63 – 66) (*n = 4, n_E =6*)	2.7 (1 – 6) (*n = 30*)	Mellen 1993[B,O], Stehlik 2003
Leopard Cat	**Pallas' cat** (*Otocolobus manul*)	9.0 – 10.0*	2.5 – 5.0*	Poly	Jan - April (NH)	14.3 (7 – 21) (*n = 6, n_E = 20*)	? (1 – 12) (*n = 6, n_E = 20*)	1.0 – 17.0 (Follicular waves may overlap) (*n = 6, n_E = 20*)	Occasional	49.8 (30 – 60) (*n = 4, n_E = 7*)	70.0 (69 – 71) (*n = 2, n_E = 2*) 80.0 – 85.0	3.5 (3 – 4) (*n = 2*)	Mellen 1993[B,O], Brown *et al.* 2002[E,O]
	Rusty-spotted cat (*Prionailurus rubiginosus*)	~12.0	1.0 – 1.1*	Poly	NS	-	5.6$^{\mu}$ (1 – 11) (*n = ?, n_E = 50*)	-	-	-	68.8$^{\mu}$ (64 – 71) (*n = ?, n_E = 26*)	1 – 2 (*n = 58*)	Mellen 1993[B,O], Dmoch 1997[R]
	Asian spotted/leopard cat (*Prionailurus bengalensis*)	8 – 12*	0.6 – 4.5*	-	All year round but peak Feb - April	-	-	-	-	-	71.5 (70 – 73) (*n = 3, n_E = 5*)	1 – 2 (*n = 41*)	Brown et al. 1994[E], Okamura et al. 2000[O], Adachi et al. 2010[E], Dehnhard et al. 2012[E]
	Fishing cat (*Prionailurus viverrinus*)	15.0 (*n = 1*)	5.1 – 6.8*	Poly	NS	21.3$^{\mu}$ (12 – 43) (*n = 18, n_E = 144*)	6.1$^{\mu}$ (4 – 8) (*n = 19, n_E = 175*)	15.2 (8 – 39) ‡	Regular	30.8$^{\mu}$ (18 – 41) (*n = 15, n_E = 41*)	69.1 (65 – 76) (*n = 6, n_E = 7*)	1 - 4*	Mellen 1993[B,O], Santymire et al. 2011[E], Dehnhard et al. 2012[E], Fazio 2016[E]
	Flat-headed cat (*Prionailurus planiceps*)	-	1.5 – 1.9*	-	-	-	-	-	-	-	~56.0*	1 – 2* (*n = 3*)	
Puma	**Puma/mountain lion** (*Puma concolor*)	~18.0*	22.7 – 57.0*	Poly	NS	19.8 (17 - 25) (*n = 3, n_E = 6*) ~23.0[(RdP 2012)]	~8.0[(RdP 2012)] 1.0 – 16.0*	11.8 (9 – 17) ‡	-	? (45 – 50) (*n = 2, n_E = 3*)	89.3 (81 – 95) (*n = 3, n_E = 3*) 84.0 – 98.0[(RdP 2012)]	1 – 6*	Bonney et al. 1981[E,L], Dehnhard et al. 2012[E]
	Jaguarondi/Eyra cat (*Puma yagouaroundi*)	17.0 – 26.0*	3.5 – 7.0*	Poly	-	53.6 ± 2.4 (*n = ?, n_E = 8*)	3.2 ±0.8 (*n = ?, n_E = 6*)	50.4 ‡	-	-	72.0 – 75.0[(RdP 2012)]	1 – 4*	Mellen 1993[B,O]
	Cheetah (*Acinonyx jubatus*)	21.0 – 24.0*	21.0 – 51.0*	Poly	NS	13.2$^{\mu}$ (3 – 30) (*n = 28, n_E = 359*)	4.1$^{\mu}$ (1 – 9) (*n = 21, n_E = 239*)	9.8 (3 – 29) ‡	Rare	52.4$^{\mu}$ (38 – 62) (*n = 18,n_E = 18*)	~93.0 (90 – 98) (*n = ?, n_E = 260*)	3.2 (1 – 8) (*n = 93*)	Asa et al. 1992[B,V], Laurenson et al. 1992[O], Brown et al. 1994[W], Graham et al. 1995[E], Brown et al. 1996[E], Marker & Dickman 2003[O], Terio et al. 2003[E], Borque et al. 2005[E], Augustus et al. 2006[O], Bertschinger et al. 2008[E,O], Adachi et al. 2011[E], Kinoshita et al. 2011b[E]
Lynx	**Iberian lynx** (*Lynx pardinus*)	24.0 – 36.0*	8.7 – 10.0*	Mono	Jan – Feb	-	2.0 – 5.0 (*n = ?, n_E = ?*)	-	Occasional	>2 years † (*n = ?, n_E = ?*)	~ 66.0 † (*n = 5, n_E = 5*)	3.0 (2 – 4) (*n = 16*)	Palomares et al. 2005[O], Göritz et al. 2009[E,U], Jewgenow et al. 2009[E], Dehnhard et al. 2012[E], Jewgenow et al. 2014[R]
	Eurasian lynx (*Lynx lynx*)	22.0 – 24.0*	13.0 – 21.0*	Mono	Jan - April	-	2.0 – 10.0 (*n > 4, n_E > 4*)	-	Occasional	>2 years † (*n = 11, n_E = 11*)	70.0 (66 – 72) † (*n = 6, n_E = 6*)	2.0 (1 – 4) (*n = 150*)	Kvam 1991[H], Henriksen et al. 2005[O], Göritz et al. 2009[E,U], Dehnhard et al. 2012[E], Jewgenow et al. 2014[R], Painer et al. 2014[E,U]
	Canadian lynx (*Lynx canadensis*)	10.0 - 23.0*	5.0 – 11.8*	Mono	Jan – Feb	-	-	-	Occasional	>2 years † (*n = 10, n_E = 10*)	(60 – 65) † (*n = 10, n_E = 10*)	1 – 8, usually 4 - 5*	Göritz et al. 2009[E,U], Fanson et al. 2010[E], Jewgenow et al. 2014[R]
	Bobcat (*Lynx rufus*)	9.0 – 12.0*	3.6 – 15.7*	Poly	Jan - June	~ 44.0 (*n = ?, n_E = ?*)	2.0 (*n = ?, n_E = ?*)	~ 42.0 ‡	Occasional	-	65.8 (62 – 70) (*n = 36, n_E = 36*)	2.5$^{\mu}$ (1 – 7) (*n = 185*)	Crowe 1975[H], Fritts & Sealander 1978[H], Parker & Smith 1983[H], Stys & Leopold 1993[O], Göritz et al. 2009[E,U], Jewgenow et al. 2014[R]

Lineage	Species	Age of female sexual maturity (Months)	BWT of adult female (kg)	Cyclicity (poly/mono-oestric)	Breeding season	Length of anovulatory oestrous cycle (Days)	Duration of oestrus (Days)	Interoestrus interval (Days)	Occurrence of spontaneous ovulation	Dioestrus		Litter size (range)	References
										Non-pregnant luteal phase (Days)	Pregnant luteal phase/Gestation (Days)		
Ocelot	**Ocelot** (*Leopardus pardalis*)	-	6.6 – 11.3*	Poly	NS	21.9$^{\mu}$ (7 – 52) (*n = 6, n_E = 108*)	4.6 (1 – 6) (*n = 7, n_E = 81*) 4.6 ± 0.6$^{(RdP\ 2012)}$	17.3 (6 – 51) ‡	Rare	? (28 – 40) (*n = 2, n_E = 7*)	79 (75 – 83) (*n = 2, n_E = 2*) 70.0 – 85.0$^{(RdP\ 2012)}$	1.2 (1 – 2) (*n = 13*)	Moreira et al. 2001^{B,L,V}, Laack et al. 2005^{O}, Putranto et al. 2006^{E}, Dehnhard et al. 2012^{E}
	Margay (*Leopardus wiedii*)	-	2.3 – 3.5*	Poly	NS	17.6$^{\mu}$ (11 – 25) (*n = 2, n_E = 32*)	? (1 – 6) (*n = 2, n_E = 32*) 4.0 – 10.0$^{(RdP\ 2012)}$	(10 – 24) ‡	Occasional	35.7$^{\mu}$ (30 – 60) (*n = 2, n_E = 9*)	83.0 (*n = 1, n_E =1*) 81.0 – 84.0$^{(RdP\ 2012)}$	Usually 1, rarely 2*	Moreira et al. 2001 ^{B,L,V}, Dehnhard et al. 2012^{E}, Moreira et al. 2007^{E},
	Andean mountain cat (*Leopardus jacobita*)	-	3.0 – 7.0*	-	-	-	-	-	-	-	-	1- 2*	
	Pampas cat/Colocolo/Pantanal cat (*Leopardus colocolo*)	-	1.7 – 3.7*	-	-	-	-	-	-	-	80.0 – 85.0*	1 – 3*	
	Geoffroy's cat (*Leopardus/Oncifelis geoffroyi*)	18.0 – 22.0 (*n = 3*)	2.6 – 4.9*	Poly	NS	5.8 (1 – 12) (*n=11, n_E=18*) ~20.0$^{(RdP\ 2012)}$	2.5 ± 0.5 (*n = 2, n_E = 2*) 2.5±0.5$^{(RdP\ 2012)}$	3 mean durations 10, 34, 60 (*n=10, n_E=15*)	-	-	66.0 – 72.0 (*n = 7, n_E = 7*) 72.0 - 76.0$^{(RdP\ 2012)}$	1 – 4 (*n = 5*)	Mellen 1993^{B,O}, Foreman 1997^{B} Dehnhard et al. 2012^{E}
	Güiña/Kodkod (*Leopardus guigna*)	?	1.3 – 2.1*	-	-	-	-	-	-	-	72.0 – 78.0*	1 – 3*	
	Tigrina/Oncilla (*Leopardus tigrinus*)	?	1.5 – 3.2*	Poly	NS	16.7$^{\mu}$ (11 – 27) (*n = 5, n_E = 44*)	2.5 (1 – 6) (*n = 2, n_E = 23*) 3.0 - 9.0$^{(RdP\ 2012)}$	14.2 (10 – 26) ‡	Rare	40.5 (40 – 41) (*n = 2, n_E = 2*)	71.0 (*n = 2, n_E = 2*) 73.0 – 78.0$^{(RdP\ 2012)}$	1 – 2*	Moreira et al. 2001 ^{B,L,V}, Moreira et al. 2007^{E}, Dehnhard et al. 2012^{E}
Caracal	**Caracal** (*Caracal caracal*)	7 – 10	6.2 – 15.9*	Poly	NS but peak season is Aug - Jan (SH)	18.9$^{\mu}$ (~10 – 54) (*n =18, n_E = 24*)	4.5$^{\mu}$ (3 – 6) (*n = 21, n_E = 21*)	15.3 (~13 – 51) ‡	-	47.5 (47 – 48) (*n = ?, n_E = 2*)	78.8$^{\mu}$ (78 – 81) (*n = 6, n_E =6*)	2.2 (1 – 4) (*n =15*)	Bernard & Stuart 1987^{B,H,O}, Mellen 1993^{B,O}, Graham et al. 1995^{E}, Dehnhard et al. 2012^{E}
	African golden cat (*Profelis/Caracal aurata*)	~11*	5.3 – 8.2*	-	-	-	-	-	-	-	75.0 (*n = 1, n_E =1*)	1 – 2*	Bahaa-el-din et al. 2015^{R}
	Serval (*Leptailurus/Caracal serval*)	15 – 16	6.0 – 12.5*	-	-	-	4.0 (*n = 1, n_E = 1*)	-	-	-	78.0 (*n = 1, n_E = 1*) 65.0 – 75.0*	2.5 (1 – 6)*	Mellen 1993^{B,O}, Dehnhard et al. 2012^{E}
Bay Cat	**Bay cat** (*Pardofelis/Catopuma badia*)	-	~2.0*	-	-	-	-	-	-	-	-	-	
	Timminck's/Asiatic golden cat (*Pardofelis temminckii*)	18.0 – 24.0*	~8.5*	Poly	NS	39.0 (*n = 1, n_E = 1*)	6.0 (*n = 1, n_E = 2*)	33.0 ‡	-	-	84.0 (*n = 1, n_E = 1*) 78.0 – 80*	1 – 3*	Mellen 1993^{B,O}, Lueders et al. 2014^{E}
	Marbled cat (*Pardofelis marmorata*)	21.0 -22.0*	2.5 – 5.0*	-	-	-	-	-	-	-	66.0 – 82.0*	2.0 (1-3)* (*n = 3*)	
Panthera	**Lion** (*Panthera leo*)	30.0 – 36.0*	110.0 – 168.0*	Poly	NS	17.9$^{\mu}$ (8 – 56) (*n = 27, n_E = 76*)	3.2 $^{\mu}$ (1 – 9) (*n = 46, n_E >167*)	14.7 (7 – 55) ‡	Occasional	46.3$^{\mu}$ (14 – 55) (*n = 16, n_E =33*)	112.7$^{\mu}$ (102 – 120) (*n = 17, n_E = 31*)	1.6 (1 – 4) (*n = 20*)	Schmidt et al. 1979^{E,V}, Schramm et al. 1994^{E}, Graham et al. 1995^{E}, Tefera 2003^{B,O}, Haas et al. 2005^{R}, Umapathy et al. 2007^{E}, Putman et al. 2015^{E}
	Jaguar (*Panthera onca*)	24.0 – 30.0*	36.0 – 100.0*	Poly	April – Sept. (Eq.) Though oestrus observed year-round	55.9 $^{\mu}$ (31 – 76) (*n = 2, n_E = 11*) 47.2 ± 5.4$^{(RdP\ 2012)}$	6.5 ± 0.3 (*n = 7, n_E =194*) 12.0 (7 – 15) (*n = 1, n_E = 7*) 12.0 ± 1.0$^{(RdP\ 2012)}$	43.9 (24 – 69) ‡	Occasional	29.6 (14-67)$^{(spontaneous)}$ (*n = 5 , n_E = 8*) 24.4 (20-33)$^{(induced)}$ (*n = 2 , n_E = 3*)	98.0 ± 0.0 (*n = 2, n_E = 2*) 90.0 – 111.0$^{(RdP\ 2012)}$	2.0 (1 – 4)*	Wildt et al. 1979^{B,L}, Putranto et al. 2006^{E}, Barnes et al. 2016^{E}, Gonzalez et al. 2017^{E}
	Leopard (*Panthera pardus*)	24.0 – 28.0*	17.0 – 42.0*	Poly	NS	23.8 (7 – 42) (*n = 3, n_E = 33*)	5.0 (4 – 7^{IQ}) (*n = 7, n_E = 156*)	20 (11 – 30) (*n = 7, n_E = 110*)	Occasional	42.6$^{\mu}$ (~34 – 56) (*n = 6, n_E = 10*)	96.9$^{\mu}$ (95 – 105^{IQ}) (*n = 4, n_E = 8*)	1.9$^{\mu}$ (1 – 3) (*n = 145*)	Schmidt et al. 1988^{E}, van Dorsser et al. 2007^{E}, Dehnhard et al. 2012^{E}, Balme et al. 2013^{R}
	Tigers (*Panthera tigris*)	~36.0*	75.0 – 177.0*	Poly	Feb - June (NH)	24.5$^{\mu}$ (6 – 148) (*n = 31, n_E = 98*)	4.3$^{\mu}$ (2.5 – 10) (*n = 18 , n_E = 33*)	17.5 (1 – 145) ‡	Rare	34.5 ±0.57 (*n = 4, n_E = 4*)	108.1 (98 – ~112) (*n = 75, n_E = 133*)	2.6 (1 – 5) (*n = 751*)	Seal et al. 1985^{E}, Kerley et al. 2003^{O}, Graham et al. 2006^{E,L}, Putranto et al. 2006^{E}, Putranto et al. 2007^{E}, Dehnhard et al. 2012^{E}, Groot 2013, Singh et al. 2013^{O}, Saunders et al. 2014, Singh et al. 2014^{O}, Gu et al. 2016^{O}
	Snow leopard (*Panthera uncia*)	25.0 – 96.0 (*n = 41*)	21.0 – 53.0*	Poly	Dec - April (NH)	14.3$^{\mu}$ (5 – 42) (*n = 15, n_E = 159*)	4.3 (1 – 19) (*n = 11, n_E = 145*)	19.2 (6 – 41) ‡	Rare	47.8 (11 – 72) (*n = 12, n_E= 16)*	93.4 (50 – 127) (*n = ?, n_E = 67*)	1 – 4 (*n = 203*)	Blomqvist & Sten 1982^{O}, Schmidt et al. 1993^{B,E}, Brown et al. 1994^{E}, Graham et al. 1995^{E}, Kinoshita et al. 2011a^{E}, Reichert-Stewart et al. 2014^{B,E}
	Clouded leopards (*Neofelis nebulosa*)	21.8 (17 – 28) (*n = 28*)	10.0 – 11.5*	Poly	Nov - July (Eq.)	28.0 (10 – 55) (*n = 42, n_E = 107*)	5.2 (1 – 17) (*n = 42, n_E = 237*)	22.8 (1 – 54) ‡	Regular	47.5 (35 – 58) (*n = ? , n_E = 21*)	93.0 (85 – 121) (*n = 23, n_E = 63*)	2.5 (1 – 5)*	Yamada & Durrant 1989^{O}, Brown et al. 1994^{E}, Brown et al. 1995^{E}

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Appendix 1c: Andrews *et al.* (2019) Supplement 3 (S3) – Data on the ejaculate characteristics of felids

A summary of the existing literature (93 publications) on the ejaculate traits of the 38 extant felid species. The felid lineages and associated species are based on Johnson et al. (2006). All values are presented as a mean ± SEM unless indicated as a weighted mean (μ), average of mean values (AV.μ), mean and range (MR), or median and interquartile range (MIQ). The male felids used in each of the studies cited are described as either captive (C), wild (W), wild bred but captive (WB,C), or privately owned (P). Males are also categorised according to sperm quality (normospermic (N) or teratospermic (T)), genetic diversity (low genetic diversity (LGD) or high genetic diversity (HGD)), or based on age. The majority of ejaculates were collected by electroejaculation, with artificial vagina (AV), epididymal sperm from castrated testis (EP), urethral catheterisation (UC) also used. These methods are described in detail in Appendix S4, which also describes the assessment of ejaculate and sperm characteristics. [A] progressive motility (PM) is a measure of the type of movement exhibited by spermatozoa: (0) no movement, (1) poor lateral movement with minimal linear movement, (2) moderate lateral movement with occasional linear movement, (3) slow linear movement, (4) linear movement, and (5) rapid linear movement. The percentage of motile sperm and PM was used to calculate the sperm motility index (SMI): [B] SMI = (% motile sperm + (20* progressive motility))/2. Many publications did not report a SMI, thus many of SMIs reported in the table were calculated from reported mean percentage of motile sperm and PM score. Calculated SMI's are indicated by †. Similarly, some of the sperm concentrations in the table below have been calculated from mean total number of sperm in the ejaculates assessed and the ejaculate volume; these values are indicated by ‡. Primary morphological abnormalities (1º abnorm.) included abnormal mid-piece, acrosomal defects, macro- or micro-cephalic, mitochondrial sheath aplasia, polycephalic, polyflagellate, and tightly coiled flagellum. Secondary morphological abnormalities (2º abnorm.) included bent mid-piece with or without cytoplasmic droplet, bent flagellum with proximal and distal cytoplasmic droplets, bent flagellum without cytoplasmic droplet, detached head, detached flagellum, and spermatids. Other abbreviations used include breeding season (BS), review article (R) and testosterone (T).

Lineage	Species	No. males	No. ejaculates	Season/time of year	pH	Testicular volume (cm^3)	Ejaculate volume (ml)	Sperm concentration (x 10^6/ml)	Vitality Or membrane integrity (%)	Sperm motility			Acrosome intactness (%)	Morphologically abnormal sperm			Reference
										% of motile sperm	PM score[A] (0 – 5)	SMI[B]		Total (%)	1º abnorm (%)	2º abnorm (%)	
Domestic Cat	**Domestic cat** (*Felis catus*)	5^{C}	24^{AV}	-	7.4 (7.0 -8.2)	-	0.04 (0.01-0.12)	1730.0 (513-3740)	-	78.0 (35-100)	-	-	-	-	-	-	Sojka et al. 1970
		16^{C}	16	-	-	-	-	147.0 ± 39.5	-	77.0 ± 3.0	-	-	-	29.1 ± 3.7	5.8	23.3	Wildt et al. 1983
		$3^{C, N}$	18	-	-	4.3 ± 0.3	0.1 ± 0.01	167.6 ± 43.6	-	84.4 ± 5.9	4.2 ± 0.3	84.4 ± 5.2	-	28.4 ± 6.4	2.5	25.9	Howard et al. 1990
		$3^{C,T}$	18	-	-	3.7 ± 0.3	0.1 ± 0.01	361.3 ± 43.6	-	73.3 ± 5.9	3.7 ± 0.3	73.6 ± 5.2	-	64.2 ± 6.4	9.0	57.2	Howard et al. 1990
		29^{C}	29^{EP}	-	-	-	N/A	80.3 ± 15.2	-	71.2 ± 2.0	2.9 ± 0.1	64.6 †	-	49.3 ± 3.2	-	-	Goodrowe & Hay 1993
		$6^{C,N}$	6	-	-	-	0.2 ± 0.01	222.3 ± 13.1	-	82.1 ± 1.1	4.1 ± 0.1	82.1 †	-	26.3 ± 1.6	-	-	Long et al. 1996
		$6^{C,T}$	6	-	-	-	0.3 ± 0.01	199.0 ± 50.3	-	69.6 ± 8.9	3.7 ± 0.1	71.8 †	-	76.0 ± 5.0	-	-	Long et al. 1996
		$?^{AV}$	-	-	6.6 – 8.8	-	0.03 - 0.04[AV.μ]	1730.0 [AV.μ]	-	56.0 – 84.0 [AV.μ]	-	-	-	<10 – 61.8 [AV.μ]	-	-	Axnér & Linde-Forsberg 2002[R]
		?	-	-	6.6 – 8.8	-	0.08 – 0.22 [AV.μ]	12.0 - 30.0 [AV.μ]	-	56.0 – 84.0 [AV.μ]	-	-	-	<10 – 61.8 [AV.μ]	-	-	Axnér & Linde-Forsberg 2002[R]
		$?^{N}$	45	-	-	-	0.23 ± 0.06	199.0 ± 19.3	-	86.2 ± 1.7	4.2 ± 0.3	85.1 †	96.5 ± 0.4	23.8 ± 1.3	-	-	Pukazhenthi et al. 2000b[R]
		$?^{T}$	31	-	-	-	0.20 ± 0.09	215.0 ± 10.6	-	80.2 ± 1.4	3.7 ± 0.2	77.1 †	98.4 ± 0.1	75.4 ± 2.4	-	-	Pukazhenthi et al. 2000b[R]
		$3^{C,N}$	9	-	-	-	0.2 ± 0.2	172.0 ± 13.2	-	87.5 ± 3.2	-	-	-	24.8 ± 3.2	-	-	Pukazhenthi et al. 2000a
		$3^{C,N}$	9	-	-	-	0.2 ± 0.1	196.0 ± 15.3	-	83.6 ± 5.1	-	-	-	73.9 ± 3.5	-	-	Pukazhenthi et al. 2000a
		$3^{C,N}$	9	-	-	-	0.26 ± 0.1	78.3 ± 11.2	-	80.2 ± 11.2	3.8 ± 0.2	78.1 †	100.0 ± 0.0	26.6 ± 3.5	-	-	Pukazhenthi et al. 2002
		$3^{C,T}$	9	-	-	-	0.22 ± 0.0	121.3 ± 32.0	-	70.2 ± 2.5	3.0 ± 0.3	65.1 †	98.0 ± 0.2	86.1 ± 2.7	-	-	Pukazhenthi et al. 2002
		$3^{C,N}$	3	-	-	-	-	-	-	63.1 ± 9.6	-	-	86.9 ± 0.3	31.5 ± 5.5	-	-	Penfold et al. 2003
		$3^{C,T}$	3	-	-	-	-	-	-	52.5 ± 4.9	-	-	20.5 ± 5.2	78.7 ± 1.5	-	-	Penfold et al. 2003
		$10^{C,N}$	10^{EP}	-	-	1.3 ± 0.09	N/A	-	-	69.0 ± 6.8	3.2 ± 0.1	66.5 †	-	33.9 ± 2.2	-	-	Neubauer et al. 2004
		$10^{C,T}$	10^{EP}	-	-	1.8 ± 0.1	N/A	-	-	74.0 ± 4.9	3.3 ± 0.1	70.0 †	-	88.6 ± 0.8	-	-	Neubauer et al. 2004
		$10^{C,T}$	10	-	-	1.8 ± 0.1	0.1 ± 0.01	1297.0 ± 252.3	-	69.7 ± 3.1	3.6 ± 0.1	70.9 †	-	90.7 ± 2.2	-	-	Neubauer et al. 2004
		48^{C}	48	-	-	-	0.1 (0.01-0.2)[MR]	-	-	60.0 (0-95) [MR]	-	-	98.5 (92-100) [MR]	66.0 (9-99) [MR]	~4.0	~30.1	Axner & Linde Forsberg 2007
		4^{C}	7	-	-	-	0.08 ± 0.01	13.5 ± 5.4	-	85.0 ± 10.0	-	-	-	-	-	-	Chatdarong et al. 2007
		8^{P}	8	-	7.9 ± 0.14	-	0.067 ± 0.01	542.9 ± 204.3	81.4 ± 3.4	78.1 ± 3.6	4.5 ± 0.2	84.1†	92.4 ± 1.2	-	-	-	Zambelli et al. 2008
		8^{p}	8^{UC}	-	7.0 ± 0.14	-	0.012 ± 0.001	1868.4 ± 353.5	80.0 ± 3.6	78.1 ± 3.4	4.7 ± 0.2	86.1†	93.6 ± 1.0	-	-	-	Zambelli et al. 2008
		22^{C}	22^{EP}	-	-	-	-	-	-	56.8 ± 2.5	2.8 ± 0.2	55.9 ± 2.0	55.3 ± 3.5	82.2 ± 2.3	-	-	Gañán et al. 2009a
		$3^{C,N}$	15	-	-	-	0.15 ± 0.1	345.0 ± 41.0	-	-	-	80.0 ± 1.0	92.0 ± 2.0	37.4 ± 4.0	9.1	28.0	Terrell et al. 2010
		$3^{C,T}$	19	-	-	-	0.17 ± 0.1	267.0 ± 37.0	-	-	-	66.0 ± 1.0	84.0 ± 2.0	73.0 ± 4.0	21.0	52.0	Terrell et al. 2010
		2^{C}	11^{AV}	-	-	-	-	-	-	82.2 ± 2.4	3.6 ± 0.8	77.1 †	49.3 ± 2.8	~63.7[μ]	-	-	Lambo et al. 2012
		$25^{P,N}$	25^{EP}	-	-	-	N/A	-	-	80.6 ± 1.6	3.1 ± 0.1	76.3 ± 1.4	97.5 ± 0.6	25.7 ± 2.0	6.7 ± 0.9	19.0 ± 1.8	Müller et al. 2012
		$12^{P,T}$	12^{EP}	-	-	-	N/A	-	-	76.7 ± 3.7	3.4 ± 0.2	72.5 ± 3.5	90.3 ± 2.0	56.9 ± 1.4	13.5 ± 1.8	43.4 ± 2.4	Müller et al. 2012
		4^{C}	4	-	-	-	0.08 ± 0.01	287.7 ± 21.3	-	84.3 ± 3.3	3.8 ± 0.2	80.2 †	-	22.7 ± 2.5	-	-	Vick et al. 2012
		13^{C}	13^{UC}	-	-	-	0.08 ± 0.9	1880 (1183-2186)[MR]	70.0 (67-79) [MR]	60.0 (5-70) [MR]	4.2 ± 0.2	72.0 †	-	-	-	-	Cunto et al. 2015
		$8^{P,N}$	8^{EP}	-	-	2.8 ± 0.05	N/A	626.9 ± 112.4	75.2 ± 5.1	78.4 ± 5.6	-	-	-	-	-	-	Gutiérrez-Reinoso & García-Herreros 2016
		$4^{P,T}$	4^{EP}	-	-	3.5 ± 0.06	N/A	952.7 ± 184.3	70.9 ± 4.9	71.2 ± 2.36	-	-	-	-	-	-	Gutiérrez-Reinoso & García-Herreros 2016
	European wild cat (*Felis silvestris*)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	African wild cat (*Felis libyca*)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Chinese mountain cat (*Felis bieti*)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Desert/Sand cat (*Felis margarita*)	8^{C}	23	NS	8.8 ± 0.04	1.6 ± 0.1	0.20 ± 0.02	209.8 ± 38.3	-	78.3 ± 1.3	3.4 ± 0.1	72.8 ± 1.2	92.9 ± 1.0	59.6 ± 3.1	-	-	Herrick et al. 2010a
		5^{C}	18	-	-	-	-	-	-	78.6 ± 1.6	3.4 ± 0.1	73.5 ± 1.5	94.0 ± 1.0	60.0 ± 3.3	-	-	Herrick et al. 2010b
	Black-footed cat (*Felis nigripes*)	5^{C}	18	NS	8.8 ± 0.06	1.8 ± 0.1	0.25 ± 0.01	130.4 ± 23.6	-	82.5 ± 1.9	3.6 ± 0.1	77.6 ± 2.0	90.5 ± 1.9	53.3 ± 3.0	-	-	Herrick et al. 2010a
		3^{C}	12	-	-	-	-	-	-	85.0 ± 1.2	3.8 ± 0.1	80.4 ± 1.0	94.0 ± 0.8	52.8 ± 4.4	-	-	Herrick et al. 2010b
	Jungle cat (*Felis chaus*)	5^{C}	5^{UC}	-	7.1 ± 0.05	-	0.07 ± 0.01	75.1 ± 7.6	60.7 ± 2.3	77.1 ± 6.3	3.08 ± 0.3	69.4 †	-	26.2 ± 2.7	-	-	Kheirkhah et al. 2017

Lineage	Species	No. males	No. ejaculates	Season/time of year	pH	Testicular volume (cm^3)	Ejaculate volume (ml)	Sperm concentration (x 10^6/ml)	Vitality Or membrane integrity (%)	Sperm motility			Acrosome intactness (%)	Morphologically abnormal sperm			Reference
										% of motile sperm	PM score[A] (0 – 5)	SMI[B]		Total (%)	1^o abnorm (%)	2^o abnorm (%)	
Leopard Cat	**Pallas' cat** (*Otocolobus manul*)	1[C]	5	Dec-April[(BS)]	-	2.0 ± 0.2	0.2 ± 0.01	123.0 ± 16.7	-	-	-	78.5 ± 2.7	-	36.6 ± 2.0	-	-	Swanson et al. 1996b
		1[C]	5	June-Oct	-	1.6 ± 0.1	0.2 ± 0.01	3.8 ± 1.8	-	-	-	64.0 ± 1.9	-	73.4 ± 4.9	-	-	Swanson et al. 1996b
		4[C]	16	-	-	-	0.07 ± 0.02	4.0 ‡	-	-	-	71.6 ± 1.4	94.8 ± 1.0	-	-	-	Swanson et al. 2006
		3[C]	3	Dec	-	2.0 ± 0.2	0.05 ± 0.02	2.3 ± 2.3	-	-	-	-	-	68.7 ± 6.8	-	-	Newell-Fugate et al. 2007
		4[C]	4	Feb (^[T])	-	2.0 ± 0.1	0.10 ± 0.02	29.0 ± 18.3	-	-	-	64.2 ± 12.7	-	52.3 ± 8.6	-	-	Newell-Fugate et al. 2007
		3[C]	3	April	-	2.5 ± 0.3	0.16 ± 0.04	51.3 ± 35.5	-	-	-	70.8 ± 3.2	-	48.0 ± 9.5	-	-	Newell-Fugate et al. 2007
		4[C]	4	June	-	2.5 ± 0.3	0.07 ± 0.01	1.5 ± 0.6	-	-	-	57.5 ± 1.8	-	75.2 ± 4.3	-	-	Newell-Fugate et al. 2007
	Rusty-spotted cat (*Prionailurus rubiginosus*)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Asian spotted/leopard cat (*Prionailurus bengalensis*)	4[C]	24	-	-	-	0.15 ± 0.02	37.0 ± 5.4	-	73.8 ± 2.6	3.5 ± 0.1	72.3 ± 2.4	-	34.6 ± 2.0	4.2	30.4	Howard & Wildt 1990
		?	43	-	-	-	0.29 ± 0.1	55.6 ± 7.7	-	68.4 ± 2.9	3.6 ± 0.1	70.2 †	95.7 ± 0.8	31.2 ± 4.1	-	-	Pukazhenthi et al. 2000b[R]
		4[C]	4	-	-	-	0.5 ± 0.1	53.0 ± 5.8	-	77.5 ± 2.1	-	-	-	23.6 ± 2.1	-	-	Pukazhenthi et al. 2000a
		12[C]	12	-	-	1.9 ± 0.6	0.13 ± 0.02	151.9 ± 34.4	70.0 ± 3.5	73.3 ± 4.7	-	-	-	~80.4 ± 3.3	-	-	Thongphakdee et al. 2011
		2[C]	2	Nov-Jan	-	-	-	-	72.1 ± 2.1	50.0 ± 15.0	-	-	-	3.2[(n = 1)]	-	-	Tajima et al. 2016
	Fishing cat (*Prionailurus viverrinus*)	8[C]	8	-	8.5 ± 0.2	5.5 ± 0.5	0.5 ± 0.1	108.0 ± 29.0	-	73.0 ± 3.0	4.0 ± 0.2	77.0 ± 3.0	90.0 ± 2.0	66.5 ± 6.8	~41.9	~24.8	Thiangtum et al. 2006
		5[C]	5	-	-	-	-	-	-	90.0 ± 2.7	-	-	-	10.4 ± 2.7	-	-	Pinyopummin et al. 2011
	Flat-headed cat (*Prionailurus planiceps*)	4[C]	8	-	8.0	-	0.1 ± 0.02	56.7 ± 6.6	48.7 ± 5.4	56.3 ± 6.7	3.1 ± 0.1	59.2 †	30.5 ± 2.5	>60	~47.1	~26.2	Thuwanut et al. 2011
Puma	**Puma/mountain lion** (*Puma concolor*)	7[C]	7	-	-	19.0 ± 0.8	3.4 ± 0.6	22.0 ± 7.3	-	64.3 ± 6.6	3.6 ± 0.2	68.2 †	98.6 ± 1.2	73.5 ± 4.9	21.7	51.8	Wildt et al. 1988
		3[C]	3	-	-	-	1.1 ± 0.4	10.8 ± 8.2	-	43.3 ± 3.3	2.7 ± 0.2	48.7 †	~ 96.0	93.0 ± 5.5	63.2	30.7	Miller et al. 1990
		-	12	-	-	-	2.8 ± 0.5	20.2 ± 4.7	-	52.0 ± 8.0	3.5 ± 0.2	61.0 †	-	76.6 ± 3.7	-	-	Howard 1993
		30[C]	30	-	-	~21.3[µ]	2.9 ± 0.3	21.5 ± 3.2	-	53.0 ± 3.7	3.2 ± 0.1	58.5 †	-	83.5 ± 1.9	-	-	Barone et al. 1994a
		31[W]	31	-	-	-	~2.5[µ]	~17.7[µ]	-	~66.8[µ]	~3.2[µ]	65.4 †	-	~73.2	-	-	Barone et al. 1994a
		16[W (LGD)]	16	-	-	9.6 ± 1.2	0.7 ± 0.1	4.8 ± 1.4	-	38.2 ± 6.7	2.3 ± 0.3	42.1 †	-	93.5 ± 0.7	-	-	Barone et al. 1994a
		?[C]	9	-	-	-	3.3 ± 0.6	37.9 ± 10.4	-	65.5 ± 2.9	3.6 ± 0.2	68.8 †	-	-	-	-	Barone et al. 1994b
		?[(LGD)]	39	-	-	-	1.6 ± 0.2	9.3 ± 1.9	-	50.3 ± 4.0	2.7 ± 0.2	52.2 †	63.5 ± 2.2	91.4 ± 1.1	-	-	Pukazhenthi et al. 2000b[R]
	Jaguarondi (*Puma yagouaroundi*)	-	3				0.1 ± 0.1	12.5 ± 9.4	-	50.0 ± 9.9	3.5 ± 0.4	60.0 †	-	64.6 ± 14.3	-	-	Howard 1993
		-	21				0.08 ± 0.02	7.2 ± 4.0	-	57.8 ± 2.5	-	-	-	74.3 ± 4.6	-	-	Morais 2001[R]
	Cheetah (*Acinonyx jubatus*)	18[C]	40	-	-	-	~1.8[(n = 15)]	14.5 ± 1.8	-	54.0 ± 3.0	-	-	-	71.0 ± 3.7	~27.4	~43.6	Wildt et al. 1983
		20[C]	29	-	-	-	1.8 ± 0.3	27.3 ± 8.6	-	70.7 ± 3.5	3.6 ± 0.1	71.4 †	96.3 ± 1.0	70.6 ± 3.3	33.2	37.3	Wildt et al. 1987b
		8[W]	8	-	-	-	-	26.7 ± 5.8	-	63.1 ± 3.9	3.8 ± 0.2	69.6 †	98.3 ± 0.5	75.9 ± 4.4	39.0	37.0	Wildt et al. 1987b
		11[C]	15	-	-	-	1.8 ± 0.3	27.3 ± 8.6	-	69.0 ± 5.8	3.7 ± 0.2	71.5 †	98.4 ± 0.5	64.6 ± 4.9	27.5	~ 36.6	Widlt et al. 1988
		9[C]	9	-	-	-	1.1 ± 0.2	40.6 ± 21.1	-	74.4 ± 3.6	3.8 ± 0.2	75.1 ± 3.7	97.0 ± 0.8	71.6 ± 4.9	24.1 ± 4.1	47.5 ± 2.9	Donoghue et al. 1992b
		5[C]	5	-	-	-	1.6 ± 0.3	13.5 ± 2.5	-	75.0 ± 2.6	4.0 ± 0.1	77.5 †	94.5 ± 1.5	74.8 ± 3.9	30.5 ± 5.8	46.0 ± 6.5	Howard et al. 1992
		12[C]	22	-	-	-	-	11.0 ± 2.2		42.7 ± 6.7	2.4 ± 0.3[(n =21)]	45.4 †	-	66.8 ± 3.7[(n =18)]	-	-	Lindburg et al. 1993
		60[C]	60	-	-	13.9 ± 0.4	1.5 ± 0.1	29.3 ± 5.3	-	67.0 ± 2.0	3.6 ± 0.1	69.5 †	-	78.7 ± 2.0	-	-	Wildt et al. 1993
		13[WB,C]	23	-	-	9.2 ± 0.4	3.7 ± 0.4	20.4 ± 3.1	-	78.0 ± 1.4	3.7 ± 0.1	76.0 †	86.3 ± 1.6	78.3 ± 2.4	27.5	50.0	Crosier et al. 2006
		97[WB,C]	200	NS	-	10.2 ± 0.3	2.1 ± 0.1	21.9 ± 1.7	-	69.0 ± 1.1	3.3 ± 0.1	67.5 †	73.9 ± 1.4	81.6 ± 0.8	38.0	~ 53.8	Crosier et al. 2007
		?[C]	160	-	6.4 - 8.0	-	0.7 ± 0.04	32.7 ± 2.9	65.2 ± 1.5	58.1 ± 1.5	-	-	-	59.7 ± 1.4	-	-	Bertschinger et al. 2008
		8[WB,C]	21	-	-	11.1 ± 0.7	3.3 ± 0.2	36.0 ± 4.9	-	70.7 ± 1.4	3.4 ± 0.1	70.7 ± 1.4	86.6 ± 1.3	80.1 ± 2.1	-	-	Crosier et al. 2009
		22[C]	22	-	-	-	2.0 ± 0.1	50.0 ± 34.0	-	-	-	69.0 ± 1.0	92.0 ± 2.0	76.0 ± 3.0	~26.4	~42.1	Terrell et al. 2010
		?[C(on exhibit)]	124	NS	-	13.4[µ]	1.5[µ]	63.8 ± 16.2[(n = 8)]	-	66.8[µ]	3.2 ± 0.1	65.4 †	79.2 ± 4.0[(n = 8)]	79.1[µ]	~34.8[(n = 8)]	~33.1[(n = 8)]	Koester et al. 2015
		?[C(off exhibit)]	58	NS	-	13.1[µ]	1.4[µ]	19.3 ± 7.6[(n = 15)]	-	69.8[µ]	3.2 ± 0.1	66.9 †	81.7 ± 2.4[(n = 15)]	75.8[µ]	~36.3[(n = 15)]	~43.1[(n = 15)]	Koester et al. 2015
		43[C]	43	-	-	12.5 ± 0.4	-	-	-	-	-	67.0 ± 1.3	-	75.0 ± 2.0	-	-	Terrell et al. 2016
		54[W]	54	-	-	13.4 ± 0.5	-	-	-	-	-	68.0 ± 1.2	-	81.0 ± 1.0	-	-	Terrell et al. 2016
Lynx	**Iberian lynx** (*Lynx pardinus*)	5[C]	5	Nov-Dec	8.0 ± 0.01	-	0.48 ± 0.06	7.6 ± 2.2	-	73.5 ± 4.6	3.1 ± 0.1	67.3 ± 2.7	40.7 ± 2.3	76.3 ± 4.0	-	-	Gañán et al. 2009b
		4[W]	4	Nov-Dec	7.4 ± 0.26	-	0.47 ± 0.08	10.1 ± 4.1	-	58.3 ± 6.3	2.7 ± 0.32	55.8 ± 5.8	49.9 ± 9.5	74.1 ± 6.0	-	-	Gañán et al. 2010
		9[C]	9	Nov-Dec	7.8 ± 0.12	-	0.34 ± 0.04	20.5 ± 6.0	-	85.6 ± 2.3	3.3 ± 0.11	75.7 ± 2.0	68.8 ± 4.4	67.0 ± 4.3	-	-	Gañán et al. 2010
		3[C]	3	Nov-Dec	8.0 ± 0.15	-	0.45 ± 0.07	8.1 ± 3.9	-	76.7 ± 6.7	3.1 ± 0.11	69.1 ± 4.4	40.1 ± 3.5	78.3 ± 3.3	-	-	Gañán et al. 2010
		3[C]	3	Feb–April[(BS)]	7.7 ± 0.21	-	0.66 ± 0.18	20.7 ± 7.4	-	62.1 ± 14.3	2.8 ± 0.12	59.4 ± 8.2	33.1 ± 1.2	79.9 ± 1.8	-	-	Gañán et al. 2010
	Eurasian lynx (*Lynx lynx*)	4[C]	4	March [(BS)]	-	3.0 ± 0.8	0.28 ± 0.07	7.6 ± 3.6	-	57.5 ± 21.4	-	-	-	74.2 ± 10.3	-	-	Jewgenow et al. 2006
		3[C]	3	June	-	2.3 ± 0.7	0.02 ± 0.01	217.8 ± 164.0	-	50.0 ± 10.0	-	-	-	98.2 ± 0.3	-	-	Jewgenow et al. 2006
		4[C]	4	November	-	2.6 ± 0.6	0.06 ± 0.03	33.8 ± 16.8	-	30.0 ± 25.0	-	-	-	94.3 ± 3.7	-	-	Jewgenow et al. 2006
		3[C]	3	March [(BS)]	-	2.8 ± 0.8	0.30 ± 0.05	8.7 ± 4.8	-	60.0 ± 30.0	-	-	-	83.2 ± 7.2	-	-	Göritz et al. 2006
		3[C]	6	April – June	-	-	0.02 ± 0.01	45.4 ± 19.5	-	36.3 ± 11.4	-	-	-	95.6 ± 1.8	-	-	Göritz et al. 2006
		3[C]	3	November	-	1.5 ± 0.2	0.13 ± 0.02	37.5 ± 27.5	-	30.0 ± 25.0	-	-	-	92.5 ± 5.5	-	-	Göritz et al. 2006
		3[C]	6	Feb–April[(BS)]	-	6.2 ± 0.1	0.04 ± 0.01	40.2 ± 19.1[(mln/ml)]	-	29.1 ± 3.0	-	-	-	63.2 ± 1.1	-	-	Erofeeva et al. 2014
		3[C]	6	May-July	-	5.4 ± 0.09	0.01 ± 0.001	490.8 ± 177.7[(mln/ml)]	-	11.9 ± 2.7	-	-	-	68.4 ± 1.4	-	-	Erofeeva et al. 2014
		3[C]	6	Aug-Oct	-	4.5 ± 0.09	0.02 ± 0.002	-	-	3.8 ± 1.0	-	-	-	76.7 ± 1.3	-	-	Erofeeva et al. 2014
		3[C]	6	Nov-Jan	-	6.3 ± 0.2	0.06 ± 0.002	-	-	21.0 ± 1.1	-	-	-	72.0 ± 0.6	-	-	Erofeeva et al. 2014
	Canadian lynx (*Lynx canadensis*)	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-
	Bobcat (*Lynx rufus*)	4[C]	4	April[(BS)]	8.0 ± 0.2	-	0.35 ± 0.08	60.6 ± 12.4	-	67.9 ± 14.1	3.3 ± 0.5	67.0 ± 10.5	33.6 ± 4.2	78.2 ± 1.6	-	-	Gañán et al. 2009c
		4[C]	9	November	7.7 ± 0.1	-	0.36 ± 0.3	10.8 ± 2.9	-	50.5 ± 5.3	2.5 ± 0.2	49.8 ± 4.4	48.0 ± 4.2	88.4 ± 1.9	-	-	Gañán et al. 2009c

Lineage	Species	No. males	No. ejaculates	Season/time of year	pH	Testicular volume (cm^3)	Ejaculate volume (ml)	Sperm concentration (x 10^6/ml)	Vitality Or membrane integrity (%)	Sperm motility			Acrosome intactness (%)	Morphologically abnormal sperm			Reference
										% of motile sperm	PM score[A] (0 – 5)	SMI[B]		Total (%)	1° abnorm (%)	2° abnorm (%)	
Ocelot	**Ocelot** (*Leopardus pardalis*)	?	5	-	-	-	0.3 ± 0.1	28.0 ± 17.0	-	72.0 ± 12.5	4.0 ± 0.5	76.0 †	-	19.2 ± 0.9	-	-	Howard 1993[R]
		2[C]	3	-	-	-	1.8 ± 1.5	187.0 ± 143.5	-	85.0 ± 2.9	4.0 ± 2.9	82.5 †	-	32.3 ± 8.8	-	-	Swanson et al. 1996a
		-	42	-			0.6 ± 0.1	53.8 ± 17.8	-	70.4 ± 2.3	-	-	-	41.6 ± 5.8	-	-	Morais 2001[R]
		3[C]	42	-	7.5 ± 0.1	32.0 ± 1.3	1.4 ± 0.1	101.2 ± 10.6	-	81.4 ± 1.2	3.7 ± 0.1	77.5 ± 1.3	99.2 ± 0.2	17.6 ± 1.2	4.0 ± 0.4	12.7 ± 1.1	Morais et al. 2002
		3[C]	7	-	-	-	0.7 ± 0.1	190.2 ± 73.2	-	81.0 ± 3.2	3.7 ± 0.2	76.0 ± 8.5	94.0 ± 0.7	22.0 ± 02.7	4.3 ± 0.7	18.7 ± 0.7	Baudi et al. 2008
		10[C]	10	-	7.9[µ]	55.6[µ]	1.0[µ]	129.4[µ]	-	77.1[µ]	3.1[µ]	69.6 †	-	32.9[µ]	-	-	Stoops et al. 2007
		3[C]	8	-	-	-	-	-	48.9 ± 5.5	85.0 ± 2.3	4.3 ± 0.1	85.5 †	99.4	52.5 ± 4.9	14.4 ± 2.4	38.1 ± 3.0	de Araujo et al. 2015
	Margay (*Leopardus wiedii*)	-	11	-	-	-	0.2 ± 0.1	79.9 ± 28.1	-	86.0 ± 3.3	4.6 ± 0.2	89.0	-	51.5 ± 6.0	-	-	Howard 1993[R]
		-	27	-	-	-	0.3 ± 0.05	14.2 ± 5.3	-	62.8 ± 5.3	-	-	-	60.5 ± 4.6	-	-	Morais 2001[R]
		3[C]	41	-	8.3 ± 0.1	6.2 ± 0.2	0.5 ± 0.01	75.6 ± 11.0	-	73.5 ± 1.3	3.4 ± 0.1	70.5 ± 1.3	95.0 ± 0.9	42.6 ± 2.8	15.8 ± 1.5	26.8 ± 2.0	Morais et al. 2002[R]
	Andean mountain cat (*Leopardus jacobita*)	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-
	Pampas cat/ Colocolo/Pantanal cat (*Leopardus colocolo*)	-	5	-	-	-	0.3 ± 0.1	10.8 ± 5.7	-	36.7 ± 6.6	2.8 ± 0.2	26.4 †	-	34.1 ± 23.8	-	-	Howard 1993[R]
		-	2	-	-	-	0.08 ± 0.01	364.0 ± 326.0	-	81.3 ± 6.3	-	-	-	43.5 ± 0.5	-	-	Morais 2001[R]
	Geoffroy's cat (*Leopardus/Oncifelis geoffroyi*)	-	8	-	-	-	0.2 ± 0.1	300.0 ± 233.2	-	73.0 ± 4.4	4.0 ± 0.3	76.5 †	-	71.0 ± 11.5	-	-	Howard 1993[R]
		-	24	-	-	-	0.2 ± 0.03	66.5 ± 24.4	-	64.0 ± 4.7	-	-	-	53.1 ± 5.0	-	-	Morais 2001[R]
	Güiña/Kodkod (*Leopardus guigna*)	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-
	Tigrina/Oncilla (*Leopardus tigrinus*)	-	18	-	-	-	0.1 ± 0.02	78.5 ± 33.8	-	62.1 ± 5.7	-	-	-	64.4 ± 6.0	-	-	Morais 2001
		4[C]	52	-	7.6 ± 0.1	4.2 ± 0.2	0.3 ± 0.1	411.9 ± 46.3	-	71.4 ± 2.3	3.8 ± 0.1	74.1 ± 1.8	97.5 ± 0.3	40.8 ± 3.5	8.6 ± 1.6	32.2 ± 2.9	Morais et al. 2002
		4[C]	4	-	-	-	0.4 ± 0.1	242.8 ± 85.2	-	78.9 ± 1.5	3.9 ± 0.1	80.0 ± 2.0	91.0 ± 5.0	23.2 ± 0.9	5.9 ± 0.7	17.3 ± 6.5	Baudi et al. 2008
		1[C]	3	-	-	-	-	-	71.7 ± 4.2	80.0 ± 0.0	4.3 ± 0.3	83.0 †	93.4	19.0 ± 1.5	5.6 ± 1.8	13.3 ± 1.4	de Araujo et al. 2015
Caracal	**Caracal** (*Caracal caracal*)	2[C]	2	-	-	-	0.24 ± 0.04	122.0 ± 114.0	85.5 ± 2.5	-	-	-	-	12.0 ± 7.0	-	-	De Schepper 2016
	African golden cat (*Profelis/Caracal aurata*)	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-
	Serval (*Leptailurus/Caracal serval*)	5[C]	5	-	-	-	0.43 ± 0.1	236.0 ± 55.0	-	73.0 ± 1.8	3.7 ± 0.2	73.5 †	93.6 ± 1.4	36.4 ± 1.4	-	-	Pukazhenthi et al. 2002
Bay Cat	**Bay cat** (*Pardofelis/Catopuma badia*)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Timminck's/Asiatic golden cat (*Pardofelis temminckii*)	1[C]	1[UC]	-	-	-	0.096	88.4	-	70.0	-	-	-	62.0	-	-	Lueders et al. 2014
	Marbled cat (*Pardofelis marmorata*)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Panthera	**Lion** (*Panthera leo*)	8[C (LGD)]	8	-	-	-	5.9 ± 0.7	13.3 ± 2.8	-	61.0 ± 3.7	-	-	96.4 ± 0.7	66.2 ± 3.6	~25.0	~41.2	Wildt et al. 1987a
		8[W (HGD)]	8	-	-	-	9.4 ± 1.4	34.4 ± 12.8	-	91.0 ± 4.2	-	-	98.9 ± 0.3	24.8 ± 4.0	~6.3	~18.5	Wildt et al. 1987a
		9[W (LGD)]	9	-	-	-	8.5 ± 0.8	25.8 ± 11.0	-	83.0 ± 4.6	-	-	90.1 ± 0.1	50.5 ± 6.8	~14.4	~36.1	Wildt et al. 1987a
		10[W (HGD)]	10	-	-	87.8 ± 6.4	6.0 ± 0.9	12.3 ± 3.8	-	89.0 ± 2.1	4.1 ± 0.3	85.5 †	99.2 ± 0.4	28.5 ± 4.8	~9.0	~19.6	Brown et al. 1991
		6[W (LGD)]	6	-	-	69.8 ± 14.9	3.4 ± 1.2	11.8 ± 9.0	-	59.0 ± 8.0	2.9 ± 0.4	58.5 †	97.3 ± 0.7	66.1 ± 7.8	~23.1	~42.6	Brown et al. 1991
		7[C]	7	-	7.9 ± 0.3	-	3.9 ± 2.4	52.1 ± 9.5	-	63.1 ± 3.8	-	-	-	22.9 ± 3.9	~15.7	~ 6.9	Shivaji et al. 1998
		7[C]	7[UC]	-	7.2 ± 0.17[(n =3)]	-	0.42 ± 0.11	1940.0 ± 610.9	66.3 ± 5.8	84.1 ± 7.7	3.5 ± 0.4[(n =3)]	77.1 †	-	54.0 ± 17.4	-	-	Lueders et al. 2012
		16[C]	17	-	-	-	-	-	-	60.0(20-95)[MR]	1.1(0.3 – 3)[MR]	41.0 †	-	66.0 (36-89)[MR]	-	-	Luther et al. 2017
	Jaguar (*Panthera onca*)	?[C]	5	-	-	-	2.7 ± 0.6	12.0 ± 1.9	-	82.0 ± 5.8	4.1 ± 0.3	82.0 †	-	41.8 ± 11.1	-	-	Howard 1993[R]
		10[C]	10	-	-	-	7.4 ± 3.7	6.2 ± 3.0	-	62.6 ± 11.0	2.7 ± 0.5	58.4 †	96.4 ± 2.0	53.3 ± 5.8	~35.1	~18.2	Morato et al. 1998
		4[C]	28	NS	-	40.4[µ](30-61)	8.6 ± 1.3	3.9 ± 0.7	-	50.6 ± 5.8	2.2 ± 0.3	47.3 †	96.2	51.0	~33.3	~17.7	Morato et al. 1999
		?[C]	38	-	-	-	5.7 ± 1.7	13.2 ± 10.8	-	56.9 ± 9.4	3.0 ± 0.8	58.5 †	-	34.3 ± 6.7	-	-	Rodrigues da Paz, 2000
		8[C]	47	-	-	41.6 ± 0.6	8.3 ± 0.7	8.0 ± 1.7	-	64.0 ± 2.4	2.8 ± 0.1	61.0 ± 2.2	95.5 ± 0.4	50.0 ± 1.1	30.0 ± 09	20.1 ± 0.9	Morato et al. 2001
		6[W]	7	-	-	52.4 ± 3.4	4.1 ± 0.7	35.0 ± 21.3	-	73.0 ± 6.1	3.5 ± 0.2	72.0 ± 5.0	98.9 ± 0.4	26.5 ± 3.9	10.0 ± 2.6	16.0 ± 2.6	Morato et al. 2001
		10[C]	10	-	-	44.4 ± 2.0	6.6 ± 1.9	6.3 ± 2.4	-	57.0 ± 4.5	2.8 ± 0.2	56.5 ± 4.5	-	39.2 ± 3.1	-	-	Morato et al. 2004
		8[C]	40	-	-	51.4 ± 2.4	5.3 ± 0.6	13.8 ± 4.2	-	60.0 ± 7.1	3.0 ± 0.1	60.0 †	-	~76.3	53.0 ± 5.1	23.3 ± 8.9	Rodrigues da Paz et al. 2006
	Leopard (*Panthera pardus*)	4[C]	14	-	-	-	5.1 ± 0.6	46.2 ± 9.8	-	43.8 ± 5.7	3.0 ± 0.3	51.9 †	94.5 ± 2.1	79.5 ± 2.0	~43.6	~35.6	Wildt et al. 1988
		8[C]	8	-	-	-	-	12.2[µ]	-	54.4[µ]	3.2[µ]	59.2 †	87.6	80.0[µ]	~40.2[µ]	~38.5[µ]	Brown et al. 1989
		?[C]	11	-	7.4 ± 0.07	-	1.6 ± 1.3	55.8 ± 38.7	-	57.1 ± 17.0	-	-	-	28.1 ± 15.3	~18.2	~7.6	Jayaprakash et al. 2001
		8[C]	37	Dec-Feb	-	-	0.3 (0.2-0.5)[MIQ]	81.3 (30-237)[MIQ]	77.0 (72-89)[MIQ]	64.0 (50-71)[MIQ]	-	-	87.0 (75-91)[MIQ]	41.0 (33-56)[MIQ]	~19.0	~19.5	van Dorsser & Strick 2005
		8[C]	16	July-Sep	-	-	0.6 (0.3-0.8)[MIQ]	12.0 (2-67)[MIQ]	69.0 (50-83)[MIQ]	44.0 (18-58)[MIQ]	-	-	72.0 (30-88)[MIQ]	71.5 (42-83)[MIQ]	~48.0	~27.8	van Dorsser & Strick 2005
		?[(Aged 2)]	5	-	-	-	0.3 (0.2-0.6)[MIQ]	2.1 (0.9-8.7)[MIQ]	75.0 (55-77)[MIQ]	50.0 (22-56)[MIQ]	-	-	74.0 (30-84)[MIQ]	71.0(56-82)[MIQ]	~61.0	~28.0	van Dorsser & Strick 2005
		?[(Aged 3-7)]	29	-	-	-	0.4 (0.3-0.6)[MIQ]	90.8 (41-237)[MIQ]	76.0 (69-90)[MIQ]	62.0 (47-69)[MIQ]	-	-	88.0 (76-90)[MIQ]	42.0 (24-58)[MIQ]	~18.0	~25.0	van Dorsser & Strick 2005
		?[(Aged 8-16)]	19	-	-	-	0.3 (0.1-0.6)[MIQ]	33.6 (7-87)[MIQ]	74.0 (63-80)[MIQ]	67.0 (20-77)[MIQ]	-	-	76.0 (58-89)[MIQ]	59.0 (23-68)[MIQ]	~33.5	~18.0	van Dorsser & Strick 2005
		1[C]	1[UC]	-	8.0	15.9	0.55	48.5	-	70	3.3	67.5	-	32.8	20.8	12.0	Baqir et al. 2015
		6[C]	6[EE]	-	7.7 ± 0.1	-	2.0 ± 0.5	84.7 ± 22.0	52.2 ± 3.8	61.0 ± 5.8	-	-	65.1 ± 5.8	33.4 – 53.2	-	-	Thuwanut et al. 2017

Lineage	Species	No. males	No. ejaculates	Season/time of year	pH	Testicular volume (cm^3)	Ejaculate volume (ml)	Sperm concentration (x 10^6/ml)	Vitality Or membrane integrity (%)	Sperm motility			Acrosome intactness (%)	Morphologically abnormal sperm			Reference
										% of motile sperm	PM score[A] (0 – 5)	SMI[B]		Total (%)	1° abnorm (%)	2° abnorm (%)	
Panthera	**Tigers** (*Panthera tigris*)	11[C]	13	-	-	-	7.0 ± 1.3	31.9 ± 8.6	-	81.5 ± 3.7	4.0 ± 0.2	80.8 †	96.9 ± 1.8	37.5 ± 6.9	~11.0	~26.8	Wildt et al. 1988
		7[C]	11	-	-	-	7.5 ± 0.8	17.3 ± 3.9	-	85.5 ± 2.1	-	-	-	18.6 ± 2.0	-	-	Donoghue et al. 1990
		5[C]	-	-	-	-	10.3 ± 0.6[(n = 46)]	57.0 ± 7.8[(n = 49)]	57.4 ± 2.3[(n =40)]	59.3 ± 2.3[(n = 45)]	-	-	-	21.7 ± 1.7[(n = 38)]	~18.2	~3.7	Byers et al. 1990
		4[C]	4	-	-	-	5.8 ± 0.7	112.0 ± 22.5	-	87.5 ± 1.4	4.6 ± 0.1	89.5 ± 1.9	-	13.7 ± 3.1	-	-	Donoghue et al. 1992a
		16[C]	16	-	7.7 ± 0.02	-	1.4 ± 0.2	41.1 ± 5.1	-	46.9 ± 3.7	-	-	-	25.2 ± 2.9	~19.0	~3.1	Shivaji et al. 1998
		?	32	-	-	-	6.5 ± 0.4	38.8 ± 6.7	-	70.8 ± 3.1	3.5 ± 0.6	70.4 †	93.8 ± 0.1	37.9 ± 2.1	-	-	Pukazhenthi et al. 2000b[R]
		1[C]	17	-	7.5 ± 0.05	-	-	~50.7‡	86.3 ± 2.7	82.4 ± 2.8	-	-	-	8.8 ± 0.8	-	-	Fukui et al. 2013
		5[C]	5	-	-	-	1.5 ± 0.4	11.4 ± 45.3	86.9 ± 4.1	72.5 ± 1.6	-	-	-	-	-	-	Kurniani Karja et al. 2016
	Snow leopard (*Panthera uncia*)	3[C]	9	Dec – Feb[(BS)]	-	11.4 ± 1.1	1.8 ± 0.5	36.3 ± 7.7	-	-	-	77.5 ± 5.5	89.1 ± 1.5	58.7 ± 4.8	~24.3	~26.8	Johnston et al. 1994
		3[C]	9	Mar-May	-	9.5 ± 0.5	2.0 ± 0.2	38.7 ± 5.3	-	-	-	86.9 ± 1.1	91.7 ± 0.9	60.6 ± 2.6	~21.2	~33.4	Johnston et al. 1994
		3[C]	9	Jun-Aug	-	8.9 ± 0.5	1.3 ± 0.1	14.2 ± 3.1	-	-	-	69.2 ± 0.3	89.7 ± 1.2	73.6 ± 2.3	~32.2	~38.2	Johnston et al. 1994
		3[C]	9	Sep-Nov	-	8.8 ± 0.6	1.3 ± 0.1	6.9 ± 1.3	-	-	-	72.2 ± 0.2	90.9 ± 1.3	67.1 ± 2.4	~30.1	~34.7	Johnston et al. 1994
		8[C]	8	-	8.6 ± 0.1	-	2.6 ± 0.3	106.7‡	-	78.1 ± 2.1	4.3 ± 0.1	81.6 ± 1.9	94.3 ± 2.5	76.3 ± 0.3	~10.8	~26.8	Roth et al. 1994
		14[C]	17	-	8.4 ± 0.1	-	2.7 ± 0.2	12.2 - 138.1‡	-	76.3 ± 2.1	3.8 ± 0.1	76.2 †	-	43.3 ± 2.8	-	-	Roth et al. 1996
		?[C]	?	-	-	-	1.4 - 5.0	4.4 - 132.1‡	-	70.0 - 90.0	-	-	-	49.0 - 67.0	-	-	Roth et al. 1997
	Clouded leopards (*Neofelis nebulosa*)	4[C]	48	-	-	-	0.64 ± 0.03	27.5 ± 2.3	-	71.0 ± 2.1	3.9 ± 0.1	74.5 †	99.5 ± 0.4	38.9 ± 1.7	~14.1	~25.1	Wildt et al. 1986
		5[C]	5	-	-	-	1.2 ± 0.07	43.8 ± 16.7	-	72.0 ± 3.4	3.9 ± 0.3	75.0 †	81.4 ± 3.5	84.6 ± 4.5	42.7 ± 3.8	37.8 ± 5.4	Howard et al. 1996
		?	147	-	-	-	1.0 ± 0.1	37.6 ± 3.3	-	66.1 ± 1.4	3.4 ± 0.1	67.1 †	63.3 ± 2.3	84.1 ± 1.3	-	-	Pukazhenthi et al. 2000b[R]
		4[C]	4	-	-	-	1.5 ± 0.8	58.3 ± 9.3	-	74.3 ± 2.2	-	-	-	84.7 ± 2.3	-	-	Pukazhenthi et al. 2000a
		4[C]	4	-	-	-	1.05 ± 0.3	59.4 ± 2.7	-	71.7 ± 6.0	3.3 ± 0.2	69.5 †	59.9 ± 3.2	79.7 ± 3.2	-	-	Pukazhenthi et al. 2002
		5[C]	5	NS	-	-	1.02 ± 0.1	43.0 ± 2.1	-	73.2 ± 4.0	-	-	31.3 ± 2.7	81.5 ± 2.3	-	-	Pukazhenthi et al. 2006
		11[C]	22	-	7.6 ± 0.1	20.8 ± 0.7	0.4 ± 0.05	178.8 ± 35.5	82.5 ± 1.5	76.8 ± 2.0	3.4 ± 0.1	72.5 ± 1.7	41.9 ± 2.3	63.9 ± 2.0	32.8	31.2	Tipkantha et al. 2016

Appendix 1c References

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Appendix 2

Publication

Andrews, C.J., Thomas, D.G., Welch, M.V., Yapura, J., Potter, M.A., 2020. Monitoring ovarian function and detecting pregnancy in felids: a review. *Theriogenology* 157, 245-253.

Appendix 2: Publication: Andrews, C.J., Thomas, D.G., Welch, M.V., Yapura, J., Potter, M.A. (2020). Monitoring ovarian function and detecting pregnancy in felids: a review. *Theriogenology* 157, 245-253.

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Christopher Jon Andrews
Name/title of Primary Supervisor:	Assoc. Prof. David Thomas
Name of Research Output and full reference:	
Andrews C, Thomas D, Welch M, Yapura J, Potter M (2020). Monitoring ovarian function and detecting pregnancy in felids: a review. Theriogenology.	
In which Chapter is the Manuscript /Published work:	Chapter 2
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	60%
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
Chris Andrews collated the literature, analysed the data, and wrote the initial draft. The author group then completed the editing phase together.	
For manuscripts intended for publication please indicate target journal:	
This article was published in the peer-reviewed journal, Theriogenology.	
Candidate's Signature:	Christopher J Andrews Digitally signed by Christopher J Andrews Date: 2021.02.18 21:11:13 +13'00'
Date:	18-2-2021
Primary Supervisor's Signature:	David Thomas Digitally signed by David Thomas DN: cn=David Thomas, c=NZ, o=Massey University, ou=Animal Science Department, email=d.g.thomas@massey.ac.nz Date: 2021.02.19 08:56:28 +13'00'
Date:	19-02-2021

(This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis)

Appendix 2b: Andrews *et al.* (2020) Appendix 1 – Lists of publications that have used various method for monitoring the reproductive sate of female felids

A list of the publications that have used, or investigated the use of, various methods for monitoring the reproductive state of felids. Note that while effort has been made to make this list as comprehensive as possible, some publications may have been missed during the literature search and are thus not included. Hormones were quantified in serum or plasma (B), faeces (F), or urine (U) samples; luteal progesterone (L) and placenta prostaglandin $F_{2\alpha}$ (P) had also been assessed. Abbreviations: assisted reproductive technologies (ART), oestradiol (E_2), faecal oestrogen metabolites (FEM), faecal progestin metabolites (FPM), progesterone (P_4), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$).

Lineage	Species	Monitoring ovarian activity					Pregnancy detection						
							Hormone monitoring (serum, faecal or urine assays)						
		Behaviour	**E_2/FEM and/or P_4/FPM (serum, faeces or urine)**	**Vaginal Cytology**	**Ultrasound**	**Laparoscopy (including confirming ovulation and guiding ART)**	**P_4/FPM (serum, faeces or urine)**	**$PGF_{2\alpha}$ (serum, faeces or urine)**	**Relaxin (serum, faeces or urine)**	**Vaginal Cytology**	**Faecal proteins (e.g. IGJ)**	**Ultrasound**	**Laparoscopy**
Domestic Cat	**Domestic cat** (*Felis catus*)	(Michael, 1961) (Sojka *et al.*, 1970) (Paape *et al.*, 1975) (Platz *et al.*, 1978) (Wildt *et al.*, 1978) (Shille *et al.*, 1979) (Wildt *et al.*, 1979a) (Wildt *et al.*, 1981a) (Schmidt *et al.*, 1983) (Glover *et al.*, 1985) (Dresser *et al.*, 1987) (Goodrowe and Wildt, 1987) (Howard *et al.*, 1992b) (Donoghue *et al.*, 1993b) (Mellen, 1993) (Swanson *et al.*, 1994) (Roth *et al.*, 1995) (Swanson *et al.*, 1995) (Swanson *et al.*, 1997) (Tsutsui *et al.*, 2000a) (Tsutsui *et al.*, 2004b) (Chatdarong *et al.*, 2006) (da Silva *et al.*, 2006) (Chatdarong *et al.*, 2007) (Harris *et al.*, 2008) (Gimenez *et al.*, 2009) (Villaverde *et al.*, 2009) (Malandain *et al.*, 2011) (Santana *et al.*, 2012) (Mitacek *et al.*, 2015) (Topie *et al.*, 2015a) (Zambelli *et al.*, 2015)	(Paape *et al.*, 1975)[B] (Verhage *et al.*, 1976)[B] (Shille *et al.*, 1979)[B] (Shille and Stabenfeldt, 1979)[B] (Wildt *et al.*, 1981a)[B] (Chan *et al.*, 1982)[B] (Schmidt *et al.*, 1983)[B] (Shille *et al.*, 1983)[B] (Glover *et al.*, 1985)[B] (Goodrowe *et al.*, 1988a)[B] (Goodrowe *et al.*, 1988b)[B] (Concannon *et al.*, 1980)[B] (Donoghue *et al.*, 1992a)[B] (Orosz *et al.*, 1992)[B] (Graham *et al.*, 1993)[F] (Root *et al.*, 1994) (Graham *et al.*, 1995)[F] (Swanson *et al.*, 1995)[B] (Roth *et al.*, 1997b)[B] (Swanson *et al.*, 1997)[B] (Graham *et al.*, 2000)[F] (Aiudi *et al.*, 2001)[B] (Graham *et al.*, 2004)[F] (Pelican *et al.*, 2005)[F] (Chatdarong *et al.*, 2006)[B] (Chatdarong *et al.*, 2007)[B] (Genaro *et al.*, 2007)[B] (Pelican *et al.*, 2008)[F] (Villaverde *et al.*, 2009)[B] (Stewart *et al.*, 2010)[F] (Malandain *et al.*, 2011)[B] (Stewart *et al.*, 2012)[F] (Kanca *et al.*, 2014)[B]	(Sojka *et al.*, 1970) (Shille *et al.*, 1979) (Mills *et al.*, 1979) (Aiudi *et al.*, 2001) (Zambelli *et al.*, 2004a) (Chatdarong *et al.*, 2006) (Chatdarong *et al.*, 2007) (Gimenez *et al.*, 2009) (Malandain *et al.*, 2011) (Kanca *et al.*, 2014) (Mitacek *et al.*, 2015) (Zambelli *et al.*, 2015)	(Gunzel-Apel *et al.*, 1998) (Malandain *et al.*, 2011) (Zambelli *et al.*, 2015)	(Platz *et al.*, 1978) (Wildt *et al.*, 1978) (Shille and Stabenfeldt, 1979) (Wildt *et al.*, 1979a) (Wildt *et al.*, 1981a) (Schmidt *et al.*, 1983) (Shille *et al.*, 1983) (Dresser *et al.*, 1987) (Goodrowe and Wildt, 1987) (Goodrowe *et al.*, 1988a) (Goodrowe *et al.*, 1988b) (Donoghue *et al.*, 1992a) (Howard *et al.*, 1992b) (Orosz *et al.*, 1992) (Donoghue *et al.*, 1993b) (Roth *et al.*, 1994) (Swanson *et al.*, 1994) (Roth *et al.*, 1997b) (Swanson *et al.*, 1997) (Graham *et al.*, 2000) (Graham *et al.*, 2004) (Pope *et al.*, 2006) (Villaverde *et al.*, 2009) (Pelican *et al.*, 2010) (Lambo *et al.*, 2012) (Pope *et al.*, 2012a) (Santana *et al.*, 2012) (Stewart *et al.*, 2012) (Conforti *et al.*, 2013) (Swanson *et al.*, 2014) (Stewart *et al.*, 2015)	(Verhage *et al.*, 1976)[B] (Concannon *et al.*, 1980)[B] (Chan *et al.*, 1982)[B] (Schmidt *et al.*, 1983)[B] (Glover et al., 1985)[B] (Hammer and Howland, 1991)[B] (Graham *et al.*, 1993)[F] (Chatdarong *et al.*, 2007)[B]	(Siemieniuch *et al.*, 2014)[B,P]	(Stewart and Stabenfeldt, 1985)[B] (Addiego *et al.*, 1987)[B] (Harris *et al.*, 2008)[B,U] (van Dorsser *et al.*, 2006)[B,U] (van Dorsser *et al.*, 2007a)[B,U] (DiGangi *et al.*, 2010)[B] (Braun *et al.*, 2012)[N/A]	-	-	(Davidson *et al.*, 1986) (Beck *et al.*, 1990) (Pope *et al.*, 1994) (Tsutsui *et al.*, 2000a) (Tsutsui *et al.*, 2000b) (Aiudi *et al.*, 2001) (Zambelli *et al.*, 2002) (Tsutsui *et al.*, 2003) (Tsutsui *et al.*, 2004a) (Zambelli *et al.*, 2004b) (van Dorsser *et al.*, 2006) (Zambelli and Prati, 2006) (Harris *et al.*, 2008) (Hildebrandt *et al.*, 2009) (Pope *et al.*, 2012b) (Santana *et al.*, 2012) (Conforti *et al.*, 2013) (Monteiro *et al.*, 2013) (Swanson *et al.*, 2014) (Gatel *et al.*, 2015) (Illanes *et al.*, 2015) (Mitacek *et al.*, 2015) (Topie *et al.*, 2015a) (Topie *et al.*, 2015b) (Zambelli *et al.*, 2015)	(Chan *et al.*, 1982)
	European/Scottish wildcat (*Felis silvestris*)	-	-	-	-	-	-	-	-	-	-	-	-
	African wildcat (*Felis libyca*)	-	-	-	-	-	-	(Dehnhard *et al.*, 2012)[F]	-	-	-	-	-
	Chinese mountain cat (*Felis bieti*)	-	-	-	-	-	-	-	-	-	-	-	-
	Desert/Sand cat (*Felis margarita*)	(Mellen, 1993)	(Herrick *et al.*, 2010a)[F]	-	-	(Herrick et al., 2010a) (Herrick et al., 2010b)	(Herrick *et al.*, 2010a)[F]	(Dehnhard *et al.*, 2012)[F]	(Harris *et al.*, 2008)[B,U]	-	-	-	-
	Black-footed cat (*Felis nigripes*)	-	(Herrick *et al.*, 2010a)[F] (Metrione *et al.*, 2019)[F]	-	-	(Herrick et al., 2010a) (Herrick et al., 2010b) (Pope *et al.*, 2012a)	(Herrick *et al.*, 2010a)[F] (Metrione *et al.*, 2019)[F]	(Metrione *et al.*, 2019)[F]	-	-	-	(Metrione *et al.*, 2019)	-
	Jungle cat (*Felis chaus*)	(Mellen, 1993)	-	-	-	-	-	-	-	-	-	-	-
Leopard cat	**Pallas' cat** (*Otocolobus manul*)	(Mellen, 1993)	(Brown *et al.*, 2002)[F]	-	-	(Brown *et al.*, 2002) (Swanson *et al.*, 2016)	(Brown *et al.*, 2002)[F]	-	(Harris *et al.*, 2008)[B,U]	-	-	-	-
	Rusty-spotted cat (*Prionailurus rubiginosus*)	(Mellen, 1993)	-	-	-	-	-	(Dehnhard and Jewgenow, 2013)[F]	-	-	-	-	-
	Asian spotted/leopard cat (*Prionailurus bengalensis*)	-	(Brown *et al.*, 1994)[F] (Adachi *et al.*, 2010)[F] (Tajima *et al.*, 2016)[F,B]	(Tajima *et al.*, 2016)	-	(Goodrowe *et al.*, 1989)	(Brown *et al.*, 1994)[F] (Adachi *et al.*, 2010)[F] (Tajima *et al.*, 2016)[F,B]	(Dehnhard *et al.*, 2012)[F] (Dehnhard *et al.*, 2017)[F,U]	-	-	-	(Tajima *et al.*, 2016)	-
	Fishing cat (*Prionailurus viverrinus*)	(Mellen, 1993) (Fazio, 2016)	(Putranto *et al.*, 2006)[F] (Santymire *et al.*, 2011)[B] (Fazio, 2016)[F]	-	-	(Pope *et al.*, 2006)	(Santymire *et al.*, 2011)[F] (Fazio, 2016)[F]	(Dehnhard *et al.*, 2012)[F]	-	-	-	-	-
	Flat-headed cat (*Prionailurus planiceps*)	-	-	-	-	(Thongphakdee *et al.*, 2010)	-	-	-	-	-	-	-

Lineage	Species	Monitoring ovarian activity					Pregnancy detection						
							Hormone monitoring (serum, faecal or urine assays)						
		Behaviour	**E_2/FEM and/or P_4/FPM (serum, faeces or urine)**	**Vaginal Cytology**	**Ultrasound**	**Laparoscopy (including confirming ovulation and guiding ART)**	**P_4/FPM (serum, faeces or urine)**	**$PGF_{2\alpha}$ (serum, faeces or urine)**	**Relaxin (serum, faeces or urine)**	**Vaginal Cytology**	**Faecal proteins (e.g. IGJ)**	**Ultrasound**	**Laparoscopy**
Puma	**Puma/mountain lion** (*Puma concolor*)	-	(Bonney *et al.*, 1981)[B] (Genaro *et al.*, 2007)[B]	(Bonney *et al.*, 1981)	-	(Bonney *et al.*, 1981) (Miller *et al.*, 1990) (Barone *et al.*, 1994)	(Bonney *et al.*, 1981)[B]	(Dehnhard *et al.*, 2012)[F]	-	-	-	(Barone *et al.*, 1994)	-
	Jaguarundi/Eyra cat (*Puma yagouaroundi*)	(Mellen, 1993)	(Genaro *et al.*, 2007)[B]	-	-	-	-	-	-	-	-	-	-
	Cheetah (*Acinonyx jubatus*)	(Asa *et al.*, 1992) (Graham *et al.*, 1995) (Wielebnowski and Brown, 1998) (Wielebnowski *et al.*, 2002) (Kinoshita *et al.*, 2009)	(Asa *et al.*, 1992)[B] (Howard *et al.*, 1992a)[B] (Graham *et al.*, 1993)[F] (Brown *et al.*, 1994)[F] (Czekala *et al.*, 1994)[F] (Doi *et al.*, 1995)[B] (Graham *et al.*, 1995)[F] (Brown *et al.*, 1996)[F] (Howard *et al.*, 1997)[B] (Wielebnowski and Brown, 1998)[F] (Doi *et al.*, 2001)[B] (Wielebnowski *et al.*, 2002)[F] (Terio *et al.*, 2003)[F] (Borque *et al.*, 2005)[F] (Bertschinger *et al.*, 2008)[B] (Kinoshita *et al.*, 2009)[F] (Crosier *et al.*, 2011)[F] (Kinoshita *et al.*, 2011b)[F] (Schulman *et al.*, 2015)[B] (Crosier *et al.*, 2017)[F] (Koester *et al.*, 2017a)[F] (Koester *et al.*, 2017b)[F] (Vernocchi *et al.*, 2018)[F] (Thuwanut *et al.*, 2019)[F]	(Asa *et al.*, 1992) (Schulman *et al.*, 2015)	(Crosier *et al.*, 2011) (Schulman *et al.*, 2015)	(Wildt *et al.*, 1981b) (Goodrowe *et al.*, 1991) (Donoghue *et al.*, 1992b) (Howard *et al.*, 1992a) (Brown et al., 1996) (Howard *et al.*, 1997) (Doi *et al.*, 2001) (Bertschinger et al., 2008) (Crosier *et al.*, 2011) (Schulman *et al.*, 2015) (Crosier *et al.*, 2017) (Thuwanut *et al.*, 2019)	(Brown *et al.*, 1994)[F] (Czekala *et al.*, 1994)[F] (Doi *et al.*, 1995)[B] (Graham *et al.*, 1995)[F] (Brown *et al.*, 1996)[F] (Borque *et al.*, 2005)[F] (Adachi *et al.*, 2011)[F] (Koester *et al.*, 2017b)[F]	(Dehnhard *et al.*, 2012)[F]	(Harris *et al.*, 2008)[B,U]	(Asa *et al.*, 1992)	(Koester *et al.*, 2017b)	(Borque *et al.*, 2005)	-
Lynx	**Iberian lynx** (*Lynx pardinus*)	-	(Braun *et al.*, 2009)[U] (Göritz *et al.*, 2009)[B] (Jewgenow *et al.*, 2009)[U] (Painer *et al.*, 2014a)[B] (Pelican *et al.*, 2009)[F]	-	(Göritz *et al.*, 2009) (Painer *et al.*, 2014a)	-	(Braun *et al.*, 2009)[B, U] (Jewgenow *et al.*, 2009)[U]	(Finkenwirth *et al.*, 2010)[F] (Dehnhard *et al.*, 2012)[F] (Dehnhard and Jewgenow, 2013)[F]	(Braun *et al.*, 2009)[B, U] (Braun *et al.*, 2012)[N/A]	-	-	-	-
	Eurasian lynx (*Lynx lynx*)	(Kachamakova and Zlatanova, 2014)	(Dehnhard *et al.*, 2008)[F] (Göritz *et al.*, 2009)[B] (Carnaby *et al.*, 2012)[L] (Painer *et al.*, 2014a)[B] (Painer *et al.*, 2014b)[B]	(Painer *et al.*, 2014b)	(Göritz *et al.*, 2009) (Painer *et al.*, 2014a) (Painer *et al.*, 2014b)	-	(Dehnhard *et al.*, 2008)[F] (Painer *et al.*, 2014b)[B]	(Dehnhard *et al.*, 2012)[F] (Painer *et al.*, 2014b)[B] (Dehnhard *et al.*, 2017)[F,U]	-	-	-	-	-
	Canadian lynx (*Lynx canadensis*)	-	(Fanson *et al.*, 2010)[F]	-	-	-	(Fanson *et al.*, 2010)[F]	-	-	-	-	-	-
	Bobcat (*Lynx rufus*)	(Stys and Leopold, 1993)	(Shille *et al.*, 1991)[F] (Woshner *et al.*, 2001)[L] (Göritz *et al.*, 2009)[B]	-	-	-	-	-	-	-	-	-	-
Ocelot	**Ocelot** (*Leopardus pardalis*)	(Swanson *et al.*, 1996) (Moreira *et al.*, 2001)	(Swanson *et al.*, 1996)[B] (Moreira et al., 2001)[F] (Putranto *et al.*, 2006)[F] (Genaro *et al.*, 2007)[B] (Paz *et al.*, 2009)[B] (Lambo *et al.*, 2014)[F]	(Moreira *et al.*, 2001) (Paz *et al.*, 2010)	-	(Swanson *et al.*, 1996) (Moreira et al., 2001) (Rodrigues da Paz et al., 2005) (Paz *et al.*, 2010) (Lambo *et al.*, 2014)	-	(Dehnhard *et al.*, 2012)[F]	-	-	-	-	-
	Margay (*Leopardus wiedii*)	(Mellen, 1993) (Moreira *et al.*, 2001) (Moreira *et al.*, 2007)	(Moreira *et al.*, 2001)[F] (Genaro *et al.*, 2007)[B] (Moreira *et al.*, 2007)[F]	(Moreira *et al.*, 2001)	-	(Moreira et al., 2001)	-	-	-	-	-	-	-
	Andean mountain cat (*Leopardus jacobita*)	-	-	-	-	-	-	-	-	-	-	-	-
	Pampas cat/Colocolo/Pantanal cat (*Leopardus colocolo*)	(Callahan and Dulaney, 1997)	-	-	-	-	-	-	-	-	-	-	-
	Geoffroy's cat (*Leopardus/Oncifelis geoffroyi*)	(Mellen, 1993) (Foreman, 1997)	(Genaro *et al.*, 2007)[B]	-	-	-	-	(Dehnhard *et al.*, 2012)[F]	-	-	-	-	-
	Güiña/Kodkod (*Leopardus guigna*)	-	-	-	-	-	-	-	-	-	-	-	-
	Tigrina/Oncilla (*Leopardus tigrinus*)	(Moreira *et al.*, 2001) (Moreira *et al.*, 2007)	(Moreira et al., 2001)[F] (Genaro *et al.*, 2007)[B] (Moreira *et al.*, 2007)[F] (Paz *et al.*, 2009)[B] (Micheletti *et al.*, 2015)[F]	(Moreira *et al.*, 2001)	-	(Moreira et al., 2001) (Rodrigues da Paz et al., 2005)	-	(Dehnhard *et al.*, 2012)[F] (Dehnhard and Jewgenow, 2013)[F]	-	-	-	-	-

Lineage	Species	Monitoring ovarian activity					Pregnancy detection						
							Hormone monitoring (serum, faecal or urine assays)						
		Behaviour	E_2/FEM and/or P_4/FPM (serum, faeces or urine)	Vaginal Cytology	Ultrasound	Laparoscopy (including confirming ovulation and guiding ART)	P_4/FPM (serum, faeces or urine)	$PGF_{2\alpha}$ (serum, faeces or urine)	Relaxin (serum, faeces or urine)	Vaginal Cytology	Faecal proteins (e.g. IGJ)	Ultrasound	Laparoscopy
Caracal	**Caracal** (*Caracal caracal*)	(Bernard and Stuart, 1987) (Goodrowe *et al.*, 1991) (Graham *et al.*, 1995)	(Graham *et al.*, 1993)[F] (Graham *et al.*, 1995)[F]	-	-	(Goodrowe *et al.*, 1991) (Pope *et al.*, 2006)	(Graham *et al.*, 1993)[F]	(Dehnhard *et al.*, 2012)[F]	-	-	-	-	-
	African golden cat (*Profelis/Caracal aurata*)	-	-	-	-	-	-	-	-	-	-	-	-
	Serval (*Leptailurus/Caracal serval*)	(Mellen, 1993)	(Shille *et al.*, 1991)[F]	-	-	-	-	(Dehnhard *et al.*, 2012)[F]	-	-	-	-	-
Bay Cat	**Bay cat** (*Pardofelis/Catopuma badia*)	-	-	-	-	-	-	-	-	-	-	-	-
	Timminck's/Asiatic golden cat (*Pardofelis temminckii*)	(Mellen, 1993)	(Lueders *et al.*, 2014)[F]	-	-	-	(Lueders *et al.*, 2014)[F]	(Lueders *et al.*, 2014)[F]	-	-	-	-	-
	Marbled cat (*Pardofelis marmorata*)	-	-	-	-	(Thongphakdee *et al.*, 2010)	-	-	-	-	-	-	-
Panthera	**Lion** (*Panthera leo*)	(Schmidt *et al.*, 1979) (Schramm *et al.*, 1994) (Graham *et al.*, 1995) (Tefera, 2003) (Umapathy *et al.*, 2007) (Callealta *et al.*, 2019)	(Schmidt *et al.*, 1979)[B] (Briggs *et al.*, 1990) (Brown *et al.*, 1993)[B] (Schramm *et al.*, 1994)[B] (Graham *et al.*, 1995)[F] (Umapathy *et al.*, 2007)[F] (Kirberger *et al.*, 2011)[B] (Goeritz *et al.*, 2012)[B] (Moresco *et al.*, 2014)[B] (Putman *et al.*, 2015)[F]	(Schmidt *et al.*, 1979) (Kirberger *et al.*, 2011) (Callealta *et al.*, 2019)	(Kirberger *et al.*, 2011) (Goeritz *et al.*, 2012) (Moresco *et al.*, 2014) (Callealta *et al.*, 2019)	(Kirberger et al., 2011)	(Schmidt *et al.*, 1979)[B] (Briggs *et al.*, 1990) (Brown *et al.*, 1993)[B] (Graham *et al.*, 1993)[F] (Schramm *et al.*, 1994)[B] (Graham *et al.*, 1995)[F] (Putman *et al.*, 2015)[F]	(Dehnhard *et al.*, 2015)[F]	(Harris *et al.*, 2008)[B,U]	-	-	-	-
	Jaguar (*Panthera onca*)	(Wildt *et al.*, 1979b)	(Wildt *et al.*, 1979b)[B] (Putranto *et al.*, 2006)[F] (Genaro *et al.*, 2007)[B] (Barnes *et al.*, 2016)[F] (Gonzalez *et al.*, 2017)[F]	-	-	(Wildt et al., 1979b) (Barnes *et al.*, 2016)	(Barnes *et al.*, 2016)[F]	(Dehnhard *et al.*, 2012)[F] (Dehnhard *et al.*, 2015)[F]	-	-	-	-	-
	Leopard (*Panthera pardus*)	(Schmidt *et al.*, 1988) (van Dorsser *et al.*, 2007b)	(Schmidt *et al.*, 1988)[B] (van Dorsser *et al.*, 2007b)[F]	-	-	-	(van Dorsser *et al.*, 2007b)[F]	(Dehnhard *et al.*, 2012)[F] (Dehnhard and Jewgenow, 2013)[F]	(van Dorsser *et al.*, 2006)[B,U]	-	-	-	-
	Tigers (*Panthera tigris*)	(Graham *et al.*, 1995) (Seal *et al.*, 1985) (Groot, 2013)	(Seal *et al.*, 1985)[B] (Graham *et al.*, 1995)[F] (Crichton *et al.*, 2003)[B] (Putranto *et al.*, 2006)[F] (Graham *et al.*, 2006)[F] (Putranto *et al.*, 2007)[F] (Groot, 2013)[F] (Lambo *et al.*, 2014)[F] (Saunders *et al.*, 2014)[F]	-	-	(Donoghue *et al.*, 1990) (Donoghue *et al.*, 1993a) (Donoghue *et al.*, 1996) (Crichton *et al.*, 2003) (Graham *et al.*, 2006) (Lambo *et al.*, 2014)	(Graham *et al.*, 1995)[F] (Graham *et al.*, 2006)[F] (Putranto *et al.*, 2007)[F] (Groot, 2013)[F]	(Dehnhard *et al.*, 2012)[F] (Dehnhard *et al.*, 2015)[F]	-	-	-	-	(Donoghue *et al.*, 1996)
	Snow leopard (*Panthera uncia*)	(Schmidt *et al.*, 1993) (Reichert-Stewart *et al.*, 2014)	(Schmidt *et al.*, 1993)[B] (Brown *et al.*, 1994)[F] (Graham *et al.*, 1995)[F] (Roth *et al.*, 1997a)[B] (Kinoshita *et al.*, 2011a)[F] (Reichert-Stewart *et al.*, 2014)[F]	-	-	(Roth *et al.*, 1997a)	(Brown *et al.*, 1994)[F] (Kinoshita *et al.*, 2011a)[F] (Reichert-Stewart *et al.*, 2014)[F]	-	-	-	-	(Broder *et al.*, 2008)	-
	Clouded leopards (*Neofelis nebulosa*)	(Yamada and Durrant, 1989) (MacKinnon, 2008) (Tipkantha *et al.*, 2017)	(Brown *et al.*, 1994)[F] (Brown *et al.*, 1995)[F] (Howard *et al.*, 1996)[B] (Howard *et al.*, 1997)[B] (MacKinnon, 2008)[F] (Tipkantha *et al.*, 2017)[F]	-	-	(Brown *et al.*, 1995) (Howard *et al.*, 1996) (Howard *et al.*, 1997) (Tipkantha *et al.*, 2017)	(Brown *et al.*, 1994)[F] (Brown *et al.*, 1995)[F] (Tipkantha *et al.*, 2017)[F]	-	-	-	-	(Howard *et al.*, 1992b)	

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Appendix 2c: Andrews *et al.* (2020) Appendix 2 – Data on the reproductive steroid concentrations of female cats.

A summary of the serum/plasma oestradiol (pg/ml) and faecal oestradiol metabolites (FEM; ng/g) concentrations during anoestrus/interoestrus (basal) and oestrus in different felid species. The felid lineages and associated species are based on Johnson *et al.* (2006). Basal and luteal (i.e. peak) concentrations of serum/plasma progesterone (ng/ml) and faecal progesterone metabolites (FPM; µg/g) are also shown. Luteal progesterone concentrations are given for both non-pregnant luteal phases (NPLP) and pregnant luteal phases (PLP). All values were calculated as weighted means of the values presented by each publication and are presented as mean (minimum-maximum). Note that some values were estimated from the graphs presented in the cited publications; these publications are indicated by '⏚'. For some studies, ranges were estimated from the standard deviation; these are indicated by '*'. Some studies listed an interquartile range rather than a true range; these studies are indicated by 'IQR'. Overall sample sizes reported as '*n*=number of animals, n_S=number of samples'. While we have endeavoured to incorporate all publications available in this table, some publications may have been missed during the literature search or may have presented values that could not easily be incorporated into this analysis; hence not all of the publications listed in **Appendix 1** have been included in this table.

Lineage	Species	Plasma or Serum					Faecal metabolites					References
		Oestradiol (pg/ml)		Progesterone (ng/ml)			FEM (ng/g)		FPM (µg/g)			
					Dioestrus/luteal phase					Dioestrus/luteal phase		
		Basal	Oestrus	Basal	NPLP	PLP	Basal	Oestrus	Basal	NPLP	PLP	
Domestic Cat	**Domestic cat** (*Felis catus*)	11 (1.1-20) (*n*=119, n_S=194)	67 (21-160) (*n*=55, n_S=184)	0.7 (<0.1-3.1) (*n*=108, n_S=149)	42 (2.1-187) (*n*=170, n_S=238)	25 (13-41) (*n*=29, n_S=31)	103 (26-259) (*n*=91, n_S=120)	207 (70-535) (*n*=103, n_S=119)	4.7 (0.1-11) (*n*=91, n_S=212)	40 (7.1-135) (*n*=40, n_S=42)	-	(Paape *et al.*, 1975); (Verhage *et al.*, 1976)*; (Shille *et al.*, 1979)*; (Shille and Stabenfeldt, 1979); (Wildt *et al.*, 1981); (Chan *et al.*, 1982)*; (Schmidt *et al.*, 1983)*; (Shille *et al.*, 1983)*; (Glover *et al.*, 1985)*; (Goodrowe *et al.*, 1988a); (Goodrowe *et al.*, 1988b)*; (Donoghue *et al.*, 1992)⏚*; (Graham *et al.*, 1995)*; (Swanson *et al.*, 1995)⏚; (Roth *et al.*, 1997b)*; (Swanson *et al.*, 1997)⏚*; (Graham *et al.*, 2000)*; (Graham *et al.*, 2004)*; (Pelican *et al.*, 2005)*; (Chatdarong *et al.*, 2006)*; (Chatdarong *et al.*, 2007)*; (Genaro *et al.*, 2007)*⏚; (Pelican *et al.*, 2008)*;(Pelican *et al.*, 2010)⏚; (Stewart *et al.*, 2010)*; (Stewart *et al.*, 2012)*
	European/Scottish wildcat (*Felis silvestris*)	-	-	-	-	-	-	-	-	-	-	-
	African wildcat (*Felis libyca*)	-	-	-	-	-	-	-	-	-	-	-
	Chinese mountain cat (*Felis bieti*)	-	-	-	-	-	-	-	-	-	-	-
	Desert/Sand cat (*Felis margarita*)	-	-	-	-	-	493 (264-1344) (*n*=8, n_S=109)	1670 (1053-3572) (*n*=8, n_S=109)	1.5 (0.3-6.0) (*n*=8, n_S=109)	-	-	(Herrick *et al.*, 2010)*
	Black-footed cat (*Felis nigripes*)	-	-	-	-	-	130 (277-2617) (*n*=8, n_S=98)	1646 (1317-4279) (*n*=8, n_S=98)	3.6 (0.7-8.9) (*n*=9, n_S=99)	27 (21 - 33) (*n*=5, n_S=26)	27 (5.0 - 38) (*n*=5, n_S=5)	(Herrick *et al.*, 2010)*; (Metrione *et al.*, 2019)*
	Jungle cat (*Felis chaus*)	-	-	-	-	-	-	-	-	-	-	-
Leopard Cat	**Pallas' cat** (*Otocolobus manul*)	-	-	-	-	-	128 (44 – 213) (*n*=6, n_S=20)	574 (155-456) (*n*=6, n_S=20)	1.0 (1.0-10) (*n*=3, n_S=3)	-	~65 (*n*=3, n_S=3)	(Brown *et al.*, 2002)⏚
	Rusty-spotted cat (*Prionailurus rubiginosus*)	-	-	-	-	-	-	-	-	-	-	-
	Asian spotted/leopard cat (*Prionailurus bengalensis*)	-	-	-	-	-	615 (313-1100) (*n*=5, n_S=7)	~3921 (*n*=3, n_S=4)	6.9 (2.3-16) (*n*=2, n_S=4)	80 (55-150) (*n*=2, n_S=4)	814 (*n*=2, n_S=2)	(Brown *et al.*, 1994); (Adachi *et al.*, 2010); (Tajima *et al.*, 2016)⏚
	Fishing cat (*Prionailurus viverrinus*)	-	-	-	-	-	161 (28-1335) (*n*=18, n_S=166)	528 (149-665) (*n*=18, n_S=166)	2.7 (2.1-8.2) (*n*=17, n_S=84)	25 (13-61) (*n*=14, n_S=41)	24 (13-141) (*n*=4, n_S=7)	(Santymire *et al.*, 2011)*; (Fazio, 2016)*
	Flat-headed cat (*Prionailurus planiceps*)	-	-	-	-	-	-	-	-	-	-	-

Lineage	Species	Plasma or Serum					Faecal metabolites					References
		Oestradiol (pg/ml)		Progesterone (ng/ml)			FEM (ng/g)		FPM (µg/g)			
					Dioestrus/luteal phase					Dioestrus/luteal phase		
		Basal	Oestrus	Basal	NPLP	PLP	Basal	Oestrus	Basal	NPLP	PLP	
Puma	**Puma/mountain lion** (*Puma concolor*)	3.0 (0.6-30) (*n*=9, *nS*=9)	? (30-375) (*n*=3, *nS*=3)	3.0 (0.9-6.1) (*n*=10, *nS*=10)	? (150-300) (*n*=3, *nS*=3)	320 (*n*=1, *nS*=1)	-	-		-	-	(Bonney *et al.*, 1981); (Genaro *et al.*, 2007)*↓
	Jaguarundi/Eyra cat (*Puma yagouaroundi*)	4.0 (1.4-6.6) (*n*=3, *nS*=3)	-	1.6 (0.5-2.7) (*n*=5, *nS*=5)	-	-	-	-	-	-	-	(Genaro *et al.*, 2007)*↓
	Cheetah (*Acinonyx jubatus*)	-	430 (*n*=1, *nS*=1)	0.8 (0.1-6.2) (*n*=24, *nS*=24)	-	-	98 (2.0-86) (*n*=62, *nS*=692)	826 (27-1900) (*n*=3, *nS*=3)	0.2 (0.01-8.0) (*n*=59, *nS*=567)	98 (3.4-1364) (*n*=49, *nS*=74)	56 (3.0-482) (*n*=27, *nS*=44)	(Brown *et al.*, 1994); (Doi *et al.*, 1995); (Graham *et al.*, 1995)*; (Brown *et al.*, 1996)*; (Wielebnowski *et al.*, 2002); (Terio *et al.*, 2003); (Borque *et al.*, 2005); (Kinoshita *et al.*, 2011b); (Schulman *et al.*, 2015); (Crosier *et al.*, 2017)↓*; (Koester *et al.*, 2017a); (Koester *et al.*, 2017b)↓*; (Vernocchi *et al.*, 2018)*; (Byron *et al.*, 2019)↓
Lynx	**Iberian lynx** (*Lynx pardinus*)	3.1 (<0.1-10) (*n*=22, *nS*=22)	-	4.0 (2.1-6.4) (*n*=32, *nS*=32)	20 (15-25) (*n*=2, *nS*=2)	17 (6.9-27) (*n*=14, *nS*=14)	-	-	-	-	-	(Goeritz *et al.*, 2009)*; (Painer *et al.*, 2014a)
	Eurasian lynx (*Lynx lynx*)	0.9 (0.4-1.3) (*n*=12, *nS* =12)	-	4.1 (0.9-7.2) (*n*=21, *nS* =22)	6.9 (0.8-11) (*n*=9, *nS* =9)	75 (10-168) (*n*=5, *nS* =5)	-	-	-	-	-	(Goeritz *et al.*, 2009)*; (Painer *et al.*, 2014a);(Painer *et al.*, 2014b)
	Canadian lynx (*Lynx canadensis*)	-	-	-	-	-	354 (282-446) (*n*=19, *nS*=19)	690 (542-878) (*n*=19, *nS*=19)	-	10 (6.0-14) (*n*=3, *nS*=3)	9.2 (4.9-18) (*n*=3, *nS*=3)	(Fanson *et al.*, 2010)↓
	Bobcat (*Lynx rufus*)	-	-	-	-	-	-	-	-	-	-	-
Ocelot	**Ocelot** (*Leopardus pardalis*)	15 (<0.1-32) (*n*=12, *nS*=12)	330 (74-586) (*n*=17, *nS*=17)	1.5 (0.5-2.9) (*n*=3, *nS*=3)	19 (8.9-12) (*n*=14, *nS*=14)	-	-	-	-	-	-	(Swanson *et al.*, 1996); (Genaro *et al.*, 2007)*↓
	Margay (*Leopardus wiedii*)	7.5 (4.0-11) (*n*=3, *nS*=3)	~500 (*n*=2, *nS* =2)	-	-	-	-	3042 (1200-8800) (*n*=2, *nS*=23)	10 (<0.1-30) (*n*=2, *nS*=?)	156 (90-340) (*n*=2, *nS*=6)	-	(Genaro *et al.*, 2007)*↓; (Moreira *et al.*, 2007)↓
	Andean mountain cat (*Leopardus jacobita*)	-	-	-	-	-	-	-	-	-	-	-
	Pampas cat/Colocolo/Pantanal cat (*Leopardus colocolo*)	-	-	-	-	-	-	-	-	-	-	-
	Geoffroy's cat (*Leopardus/Oncifelis geoffroyi*)	6.0 (3.2-8.8) (*n*=2, *nS*=2)	-	-	-	-	-	-	-	-	-	(Genaro *et al.*, 2007)*↓;
	Güiña/Kodkod (*Leopardus guigna*)	-	-	-	-	-	-	-	-	-	-	-
	Tigrina/Oncilla (*Leopardus tigrinus*)	7.5 (1.9-13) (*n*=14, *nS*=14)	~250 (*n*=3, *nS*=3)	-	-		95 (10-200) (*n*=6, *nS*=~250)	1650 (250-5000) (*n*=9, *nS*=38)	8.9 (<0.1-30) (*n*=9, *nS*=~133)	352 (35-1600) (*n*=9, *nS*=15)		(Genaro *et al.*, 2007)*↓; (Moreira *et al.*, 2007)↓; (Micheletti *et al.*, 2015)↓
Caracal	**Caracal** (*Caracal caracal*)	-	-	-	-	-	~10 (*n*=2, *nS*=84)	24 (17-33) (*n*=4, *nS*=18)	~0.3 (*n*=2, *nS*=36)	2.5 (0.9-3.9) (*n*=4, *nS*=19)	2.5 (0.9-3.9) (*n*=4, *nS*=19)	(Graham *et al.*, 1993), (Graham *et al.*, 1995)*
	African golden cat (*Profelis/Caracal aurata*)	-	-	-	-	-	-	-	-	-	-	-
	Serval (*Leptailurus/Caracal serval*)	-	-	-	-	-	-	-	-	-	-	-

Lineage	Species	Plasma or Serum					Faecal metabolites					References
		Oestradiol (pg/ml)		Progesterone (ng/ml)			FEM (ng/g)		FPM (µg/g)			
					Dioestrus/luteal phase					Dioestrus/luteal phase		
		Basal	Oestrus	Basal	NPLP	PLP	Basal	Oestrus	Basal	NPLP	PLP	
Bay Cat	**Bay cat** (*Pardofelis/Catopuma badia*)	-	-	-	-	-	-	-	-	-	-	-
	Timminck's/Asiatic golden cat (*Pardofelis temminckii*)	-	-	-	-	-	~250 (*n*=1, n_S=19)	1800 (1200-2400) (*n*=1, n_S=2)	-	-	-	(Lueders *et al.*, 2014)⏚
	Marbled cat (*Pardofelis marmorata*)	-	-	-	-	-	-	-	-	-	-	-
Panthera	**Lion** (*Panthera leo*)	20 (17-23) (*n*=3, n_S=3)	ND (19-108) (*n*=3, n_S=9)	3.4 (0.2-13) (*n*=27, n_S=40)	33.4 (17-282) (*n*=12, n_S=16)	15 (9.9-144) (*n*=3, n_S=3)	61 (40-23) (*n*=3, n_S=3)	405 (112-1127) (*n*=29, n_S=51)	2.9 (0.3-5.2) (*n*=28, n_S=2631)	6.3 (0.5-28.2) (*n*=24, n_S=46)	9.5 (1.4-82) (*n*=11, n_S=33)	(Graham *et al.*, 1995)*; (Schmidt *et al.*, 1979); (Schramm *et al.*, 1994)*; (Umapathy *et al.*, 2007); (Brown *et al.*, 1993); (Goeritz *et al.*, 2012); (Moresco *et al.*, 2014); (Putman *et al.*, 2015)*
	Jaguar (*Panthera onca*)	3.9 (0.2-7.6) (*n*=6, n_S=6)	-	1.1 (<0.1-3.9) (*n*=5, n_S=17)	28.5 (12-68) (*n*=1, n_S=4)	-	1.3 (0.8-2.2) (*n*=3, n_S=3)	143 (28-74) (*n*=3, n_S=3)	18 (14-25) (*n*=3, n_S=3)	13.3 (2.5-109) (*n*=10, n_S=25)	14 (*n*=1, n_S=1)	(Wildt *et al.*, 1979); (Genaro *et al.*, 2007)*⏚; (Barnes *et al.*, 2016); (Gonzalez *et al.*, 2017)*
	Leopard (*Panthera pardus*)	-	66 (21-131) (*n*=3, n_S=19)	1.6 (<0.1-6.2) (*n*=3, n_S=131)	68 (13-98) (*n*=2, n_S=5)	-	714 (305-938) (*n*=9, n_S=234)	1436 (1281-1604) (*n*=9, n_S=156)	-	30 (5.0-60) (*n*=9, n_S=242)	64 (26-146) (*n*=9, n_S=397)	(Schmidt *et al.*, 1988)*; (van Dorsser *et al.*, 2007)[IQR]
	Tigers (*Panthera tigris*)	5.5 (0.5-15) (*n*=3, n_S=98)	48 (24-115) (*n*=3, n_S=17)	1.2 (0.2-2.8) (*n*=20, n_S=163)	-	-	205 (44-1022) (*n*=26, n_S=477)	722 (71-2503) (*n*=46, n_S=62)	0.4 (0.1-2.8) (*n*=20, n_S=339)	12.4 (2.4-51) (*n*=11, n_S=33)	8.2 (2.4-29) (*n*=7, n_S=29)	(Graham *et al.*, 1995)*; (Seal *et al.*, 1985); (Crichton *et al.*, 2003)*; (Graham *et al.*, 2006)*; (Putranto *et al.*, 2007)*
	Snow leopard (*Panthera uncia*)	7.6 (1.0-12) (*n*=2, n_S=29)	181 (37-440) (*n*=6, n_S=17)	~2.0 (*n*=2, n_S=32)	-	39 (*n*=1, n_S=1)	2133 (100-1400) (*n*=20, n_S=229)	3349 (67-13500) (*n*=20, n_S=113)	0.6 (0.1-5.4) (*n*=17, n_S=149)	18.5 (7.8-59) (*n*=11, n_S=14)	22.4 (9.5-41) (*n*=5, n_S=5)	(Graham *et al.*, 1995)*; (Schmidt *et al.*, 1993)*⏚; (Brown *et al.*, 1994); (Roth *et al.*, 1997a); (Kinoshita *et al.*, 2011a)*⏚; (Reichert-Stewart *et al.*, 2014)
	Clouded leopards (*Neofelis nebulosa*)	-	-	<1.0 (*n*=?, n_S=14)	51 (32-74) (*n*=23, n_S=27)	-	49 (20-100) (*n*=26, n_S=95)	182 (98-354) (*n*=22, n_S=72)	5.0 (2.5-6.6) (*n*=26, n_S=39)	172 (67-585) (*n*=22, n_S=22)	178 (87-345) (*n*=5, n_S=8)	(Brown *et al.*, 1994); (Brown *et al.*, 1995)*⏚; (Howard *et al.*, 1997)*

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Appendix 3

Appendix 3: The neurological pathways by which stress activates the hypothalamic-pituitary-adrenal axis

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A3.1 Introduction

Stress, defined as any predicted threat or physical challenge to homeostasis, leads to the activation of the hypothalamic-pituitary-adrenal (HPA) axis, which has an important role in mediating many of the effects of stress on the body (Miller and O'Callaghan, 2002). The HPA axis is primarily regulated by corticotrophin-releasing hormone (CRH) neurons in the paraventricular nucleus (PVN) of the hypothalamus (Miller and O'Callaghan, 2002). These neurons heavily project into the median eminence (ME) where they secrete CRH into the hypophyseal-portal blood (Vale *et al.*, 1981; Miller and O'Callaghan, 2002). Corticotrophin-releasing hormone dose-dependently stimulates corticotroph cells in the anterior pituitary to synthesise and secrete adrenocorticotrophic-releasing hormone (ACTH), which acts on cells within the adrenal cortex to promote the production and release of glucocorticoids (Rivier and Plotsky, 1986; Miller and O'Callaghan, 2002).

It has been well documented that stress leads to the activation of CRH neurons in the PVN and subsequently the release of CRH into the hypophyseal portal blood (Rivier and Plotsky, 1986; Miller and O'Callaghan, 2002). For example, there is a substantial increase in hypothalamic CRH concentrations in rat within 1 hour of restraint stress (Moldow *et al.*, 1987; Kalin *et al.*, 1994). The neurological pathways by which stress activates these CRH neurons are complex and differs between reactive (response to a homeostatic challenge) and anticipatory (perceived homeostatic challenge) stressors. Appendix 3 reviews the complex neurological pathways by which reactive and anticipatory stressors activate the CRH neurons in the PVN, and thus, the HPA axis.

A3.2 Review

A3.2.1 Reactive stressors

The brainstem receives most of the visceral afferent pathways associated with homeostasis (e.g., baroreceptors, visceral nociceptors, thermoreceptors, satiety signals, and respiratory and cardiovascular function; Ter Horst *et al.*, 1989). Thus, it not surprising that the brainstem also has a major role in mediating the effects of reactive stressors (i.e., disruptions in homeostasis) on the HPA axis (Figure A3.1).

Numerous noradrenergic neurons in the nucleus tractus solitarius (NTS), ventrolateral medulla (VLM) and locus coeruleus (LC) of the lower brainstem have been found to synapse with CRH neurons in the PVN of the hypothalamus (Figure A3.1), suggesting a role for these regions in mediating the physiological stress response (Leibowitz *et al.*, 1988; Ter Horst *et al.*, 1989; Pacak *et al.*, 1995; Herman *et al.*, 2003). Indeed, stress responses associated with somatic pain (e.g., foot shocks), physical restraint, and immune responses (elevated interleukin-1β) have all been shown to activate

noradrenergic neurons in the brainstem (specifically the NTS) of rats, followed by the release of noradrenalin (NA) into the PVN (Leibowitz *et al.*, 1988; Ter Horst *et al.*, 1989; Pezzone *et al.*, 1993; Pacak *et al.*, 1995; Palkovits *et al.*, 1999; Herman *et al.*, 2003). Corticotrophin-releasing hormone neurons in the PVN have been found to express both α_1 and β adrenergic receptors and are thus capable of responding to stress-induced increases in NA within the PVN (Rivier and Rivest, 1991). Noradrenalin appears to have a stimulatory effect on paraventricular CRH neurons (Plotsky, 1987; Herman *et al.*, 2003). The intracerebroventricular administration of NA certainly leads to a substantial increase in the concentration of CRH in the hypophyseal-portal blood, but it was not clear whether this was mediated via direct or indirect pathways (Plotsky, 1987; Herman *et al.*, 2003).

Noradrenergic neurons from the LC and NTS have also been found to project into the median amygdala and raphe nuclei, respectively (Figure A3.1; Rivier and Rivest, 1991). The dorsal raphe nuclei of the brainstem contains a high density of serotonergic perikarya that are stimulated by noradrenergic neurons from the NTS (Rivier and Rivest, 1991). The majority of serotonergic fibres that innervate the hypothalamus originate from the dorsal raphe nuclei of the brainstem, but few serotonergic axons terminate in the PVN (Herman *et al.*, 2003; Aguilera and Liu, 2012). However, serotonergic neurons from the raphe nuclei extensively inhibit the dense array of gamma-aminobutyric acid (GABA) neurons in the regions surrounding the PVN; these GABA neurons project into the PVN and dose-dependently inhibit the CRH neurons located there, thus suppression of these GABA neurons by serotonin would likely increase the activity these CRH neurons (Figure A3.1; Pan and Gilbert, 1992; Herman *et al.*, 2003; Cullinan *et al.*, 2008; Lee *et al.*, 2008; Aguilera and Liu, 2012). Indeed, a microinjection of a serotonin receptor agonist into the PVN of rats leads to an increase in the activity of the CRH neurons regulating the HPA axis (Pan and Gilbert, 1992). Serotonergic fibres from the raphe nuclei also densely innervate the amygdala, ventral subiculum of the hippocampus (vSUB), prefrontal cortex (PFC), and bed nucleus of the stria terminalis (BNST; Figure A3.1; Lowry, 2002; Herman *et al.*, 2003). As a result, it has been suggested that the serotonergic system has a role in relaying information about reactive stressors to the limbic system (Lowry, 2002; Herman *et al.*, 2003).

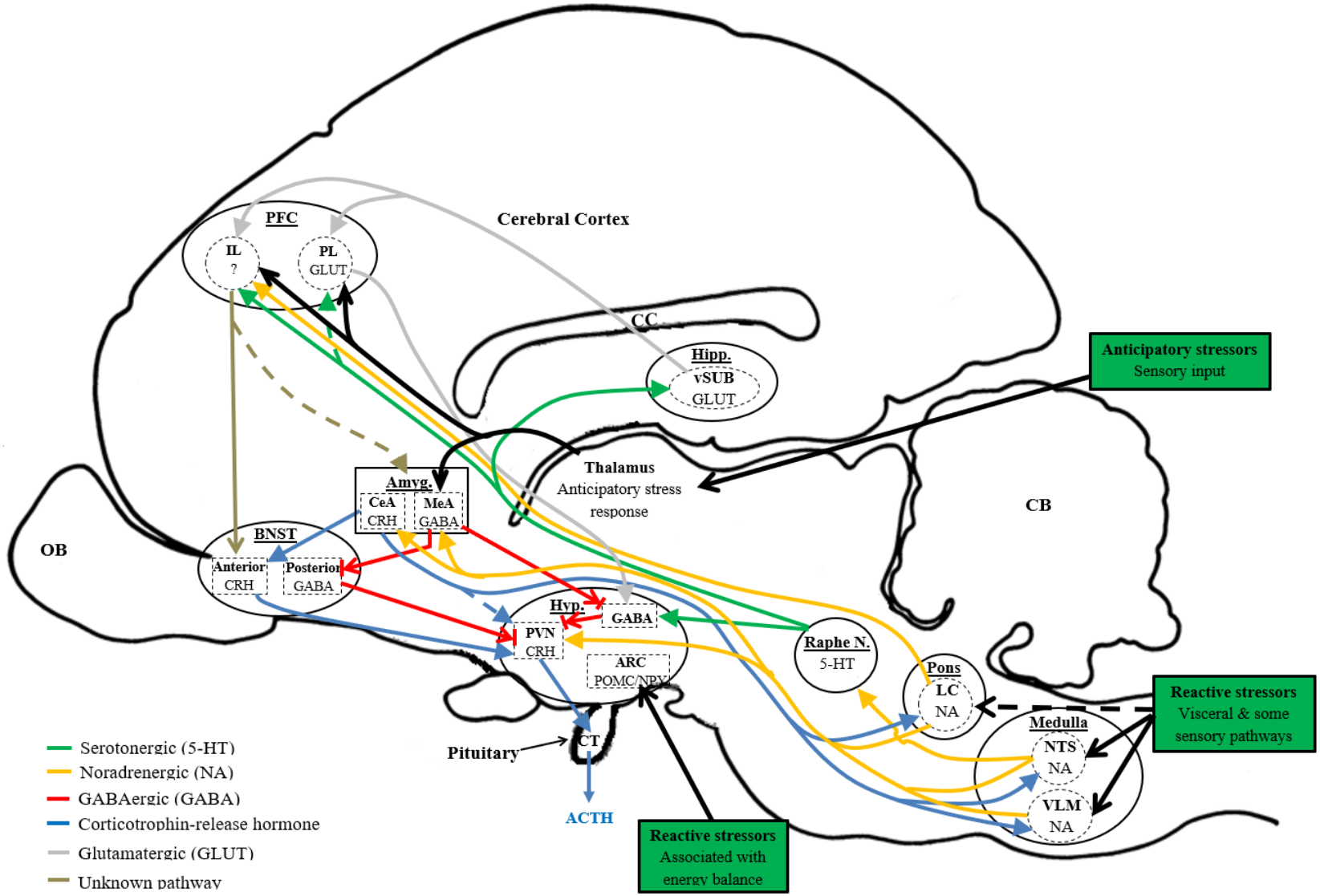

Figure A3.1 The main anatomical pathways by which reactive and anticipatory stressors activate corticotrophin-releasing hormone (CRH) neurons in the paraventricular nucleus. Regions are labelled according to the name of region/the main neurons within this region. Abbreviations: adrenocotricotrophin-releasing hormone (ACTH), arcuate nucleus of hypothalamus (ARC), bed nucleus of the stria terminalis (BNST), corticotrophin-releasing hormone neurons (CRH), central amygdala (CeA), cerebellum (CB), corpus callosum (CC), corticotroph cells (CT), gamma-aminobutyric acid neurons (GABA), glutamate neurons (Glut), hypothalamus (Hyp.), infralimbic PFC (IL), locus ceruleus (LC), median amygdala (MeA), neuropeptide Y neurons (NPY), noradrenergic neurons (NA), nucleus tractus solitaries (NTS), olfactory bulb (OB), prefrontal cortex (PFC), prelimbic PFC (PL), proopiomelanocortin neurons (POMC), raphe nuclei (raphe N.), serotonergic neurons (5-HT), ventral subiculum of hippocampus (vSUB), ventrolateral medulla (VLM).

Interestingly, reactive stressors associated with energy balance influence the activity of the HPA axis via a pathway that is largely independent of both the noradrenergic and serotoninergic systems (Figure A3.1). Instead, the arcuate nucleus (ARC) of the hypothalamus appears to have an important role in mediating the effects of stress associated with energy balance (e.g., malnutrition/starvation) on the HPA axis (Herman *et al.*, 2003). The ARC has two neuronal populations that are involved in the regulation of energy balance and food intake: (1) pro-opiomelanocortin (POMC) neurons and (2) neuropeptide Y (NPY) neurons (Woods *et al.*, 1998). Both POMC and NPY neurons in the ARC are responsive to the concentrations of insulin and leptin in the blood, which vary according to energy balance and body condition (Woods *et al.*, 1998).

Insulin and leptin stimulate POMC neurons and inhibit NPY neurons (Woods *et al.*, 1998). Excessive negative energy balance (i.e., low leptin and insulin) results in an increase in the activity of NPY neurons in the ARC, which project into the PVN and stimulate the CRH neurons regulating the HPA axis (Leibowitz *et al.*, 1988). Obesity, or an excessively positive energy balance, has also been shown to activate the HPA axis (Nieuwenhuizen and Rutters, 2008). This is more likely mediated by an increase in the activity of POMC neurons in the ARC, which heavily project into the PVN and secrete the neuropeptide hormone α-melanocyte stimulating hormone (α-MSH; Dhillo *et al.*, 2002; Herman *et al.*, 2003). In rats, α-MSH has been found to stimulate hypothalamic CRH production (*in vitro*) and increase the plasma ACTH concentrations *in vivo* (Dhillo *et al.*, 2002). Thus, it seems that high levels of NPY (negative energy balance) and POMC (positive energy balance) activate the HPA axis.

A3.2.2 Anticipatory stressors

Anticipatory stressors require the integration of past experiences (i.e., memory) and existing somatic sensory information associated with a perceived threat to homeostasis (Herman *et al.*, 2003). Therefore, the limbic system plays a critical role in mediating the effects of anticipatory stressors on the HPA axis. The thalamus has a particularly important role in the anticipatory stress response as it receives input from almost every visceral and somatic sensory pathway (Herman *et al.*, 2003). Somatic and visceral sensory information is assessed by the thalamus and relayed, if necessary, to the appropriate sensory region of the cerebral cortex or non-cortical regions of the forebrain (Herman *et al.*, 2003). In rats, an increase in neuronal activity in the paraventricular nucleus of that thalamus (PVT) has been observed in response to both anticipatory and reactive stressors (Bubser and Deutch, 1999). Neurons in the PVT do not directly innervate hypothalamic CRH neurons, but heavily innervate the pre-frontal cortex (PFC) and central amygdala (CeA; Figure A3.1; Bubser and Deutch, 1999; Herman *et al.*, 2003).

The amygdala is generally thought to have a stimulatory effect on the HPA axis (Herman *et al.*, 2003). The stimulatory effects of the amygdala on CRH neurons in the PVN are predominantly

mediated by the central (CeA) and medial amygdaloid nuclei (MeA), which chiefly contain CRH and GABA neurons, respectively (Champagne *et al.*, 1998; Herman *et al.*, 2004; Herman *et al.*, 2005). Corticotrophin-releasing hormone neurons in the CeA receive input from the NA neurons in the brainstem and are strongly activated by reactive stressors (Figure A3.1; Feldman *et al.*, 1994; Asan, 1998; Herman *et al.*, 2003; Ulrich-Lai and Herman, 2009). In contrast, the MeA contains many GABA neurons that are stimulated by thalamus (pathways unclear) in response to anticipatory stressors, which thus indirectly stimulate the HPA axis (Herman *et al.*, 2003).

Interestingly, limbic structures such as the amygdala, hippocampus, and prefrontal cortex, appear to have few direct connections with CRH neurons in the PVN of the hypothalamus (Prewitt and Herman, 1998; Ulrich-Lai and Herman, 2009). Instead, the effects of these limbic regions on the HPA axis are largely mediated by the bed nuclei of the stria terminalis (BNST; Figure A3.1; Dong *et al.*, 2001; Choi *et al.*, 2007; Ulrich-Lai and Herman, 2009; Aguilera and Liu, 2012). Corticotrophin-releasing hormone neurons in the CeA have a number of projections into the dorsomedial fusiform nuclei of the anterior BNST, a region that is concentrated with CRH neurons that project into the PVN and stimulate the CRH neurons regulating the HPA axis (Figure A3.1; Cummings *et al.*, 1983; Champagne *et al.*, 1998; Prewitt and Herman, 1998; Dong *et al.*, 2001; Choi *et al.*, 2007). The GABA neurons in the MeA project into the posterior BNST and preoptic area of the hypothalamus (POA); both of regions contain GABA neurons that project into that PVN and suppress CRH neuronal activity (Figure A3.1), thus MeA appears to stimulate the HPA axis by supressing the GABAergic inhibition of CRH neurons in the PVN (Prewitt and Herman, 1998; Herman *et al.*, 2003).

Unlike the amygdala, the hippocampus primarily has an inhibitory effect on the HPA axis (Herman *et al.*, 2003; Ulrich-Lai and Herman, 2009). The effects of the hippocampus on CRH neurons in the PVN are thought to be mediated by neurons in the ventral subiculum of hippocampus (vSUB), as lesions to this area increases baseline CRH mRNA expression in the PVN of rats (Herman *et al.*, 2003; Herman *et al.*, 2005; Ulrich-Lai and Herman, 2009). Lesions to the vSUB of rats also prolongs stress-induced increases in HPA activity, suggesting that the hippocampus has a role in terminating the stress response (Herman *et al.*, 2003; Herman *et al.*, 2005; Ulrich-Lai and Herman, 2009). The vSUB receives input from the noradrenergic and serotonergic neurons in the brainstem that are highly involved in the reactive stress response (Figure A3.1**;** Segal and Landis, 1974; Cummings *et al.*, 1983). In terms of the anticipatory stress response, however, the vSUB of the hippocampus likely receives information directly from the sensory thalamic nuclei (Ulrich-Lai and Herman, 2009). As with the amygdala, the vSUB does not directly innervate the PVN, but has an extensive network of glutamatergic (GLUT) neurons that project into the posterior BNST and various regions of hypothalamus, especially the preoptic area (POA; Cullinan *et al.*, 1993; Herman *et al.*, 2003). As mentioned previously, both the posterior BNST and POA contain GABA neurons that are known to

inhibit on CRH neurons in the PVN (Figure A3.1; Herman *et al.*, 2003). Given that GLUT neurons are generally considered to be stimulatory (Herman *et al.*, 2003), it is likely that the hippocampus supresses the HPA axis up-regulating the GABA neurons that supress CRH neurons within the PVN.

The hippocampus, most likely vSUB, also strongly innervates the prefrontal cortex (PFC; Hoover and Vertes, 2007). Given the known role of the hippocampus in memory, it is possible that the neurons from the hippocampus provide the PFC with information about past experiences (Hoover and Vertes, 2007). The PFC also receives extensive input from the midline thalamus, which likely provides the PFC with current sensory information (Bubser and Deutch, 1999; Herman *et al.*, 2003; Hoover and Vertes, 2007). It is well known the PFC plays an important role in integrating information about present and past experiences (Bubser and Deutch, 1999; Herman *et al.*, 2003; Hoover and Vertes, 2007), thus the PFC likely has an essential role in mediating the anticipatory stress response. The PFC may also contribute towards the psychological/emotional stress associated with both reactive and anticipatory stressors (Figure A3.1).

The PFC also indirectly alters the activity of the HPA axis (Ulrich-Lai and Herman, 2009). The prelimbic region of the PFC appears to have an inhibitory effect on the HPA axis, since lesions to the prelimbic region of PFC augment the stress-induced increase in the activity of CRH neurons in the PVN (Ulrich-Lai and Herman, 2009). The prelimbic region of PLC is concentrated with GLUT neurons that project into regions of the hypothalamus surround the PVN and up-regulate GABA neurons that inhibit CRH neurons in the PVN (Figure A3.1; Hurley *et al.*, 1991; Ulrich-Lai and Herman, 2009); thus, the prelimbic region of PFC inhibits the HPA axis. In contrast, the infralimbic region of the PFC appears to simulate the HPA axis (Herman *et al.*, 2003). The neurons involved in this are not known, but the infralimbic region of PLC has dense projections into the several regions of the brain (e.g., dorsomedial nucleus and POA of the hypothalamus, anterior BNST, CeA, MeA, and NTS) that are known to regulate the CRH neurons in the PVN (Hurley *et al.*, 1991; Herman *et al.*, 2005; Ulrich-Lai and Herman, 2009).

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Appendix 4

Figure: Pathways by which the hypothalamic-pituitary-adrenal (HPA) axis suppresses the hypothalamic-pituitary-gonadal (HPG) axis.

Appendix 4 - Figure: Pathways by which the hypothalamic-pituitary-adrenal (HPA) axis suppresses the hypothalamic-pituitary-gonadal (HPG) axis.

Figure A4 (on following page) summarises the pathways by which glucocorticoids (GC) and corticotrophin releasing hormone (CRH) neurons suppress the hypothalamic-pituitary-gonadal axis (HPG) axis. Red boxes indicate pathways that have a negative effect on the HPG axis. In contrast, green boxes are key intra-cellular signaling compounds/enzymes or receptors that have a positive effect on the HPG axis. Blue boxes represent cellular responses. Green lines indicate important pathways that have a stimulatory effect on the HPG axis, while red lines indicate key inhibitory pathways. Black lines indicate generally signaling pathways. Dotted lines are suggested pathways that have not yet been confirmed. Percentages represent the proportion of neurons that have receptors for that neurotransmitter or hormone. The lists below the testis and ovary boxes summarise the effects of GC and CRH on reproductive function of male and female mammals.

Other abbreviations: action potential (AP), adenylate cyclase (AC), androgen receptor (AR), anteroventral periventricular nucleus, arcuate nucleus (ARC), cyclic adenosine monophosphate response element binding protein (CRE-BP), chaperone protein (Chap.), corticotrophin release hormone (CRH), corticotrophin release hormone receptor (CRH-R), cyclic adenosine monophosphate (cAMP), desmosome junction (desmosome J.), diacylglycerol (DAG), dorsomedial nucleus (DMN), elongate spermatocyte (E), follicle stimulating hormone (FSH), follicle stimulating hormone receptor (FSH-R), G-protein coupled receptor (GPR), glucocorticoid receptor (GR), gonadotrophin inhibitory hormone (GnIH), gonadotrophin release hormone (GnRH), G protein alpha subunit – inhibitory ($G\alpha_i$), G protein alpha subunit – stimulatory ($G\alpha_S$), G protein alpha subunit q/11 – stimulatory ($G\alpha_{q/11}$) , hydroxysteroid dehydrogenase (HSD), inositol trisphosphate (IP_3), intracellular calcium (iCa^{2+}), kisspeptin (KiSS), luteinizing hormone (LH), luteinizing hormone receptor (LH-R), mitogen-activated protein kinase (MAPK), oestradiol (E_2), phospholipase C (PLC), potassium (K^+), preoptic area (POA), primary spermatocyte (I), protein kinase A (PKA), protein kinase C (PKC), round spermatocyte (R), secondary spermatocyte (II), testosterone (T), transient receptor potential cation channel (TRPC).

Appendix 4 – Pathways by which the HPA axis inhibits the HPG axis

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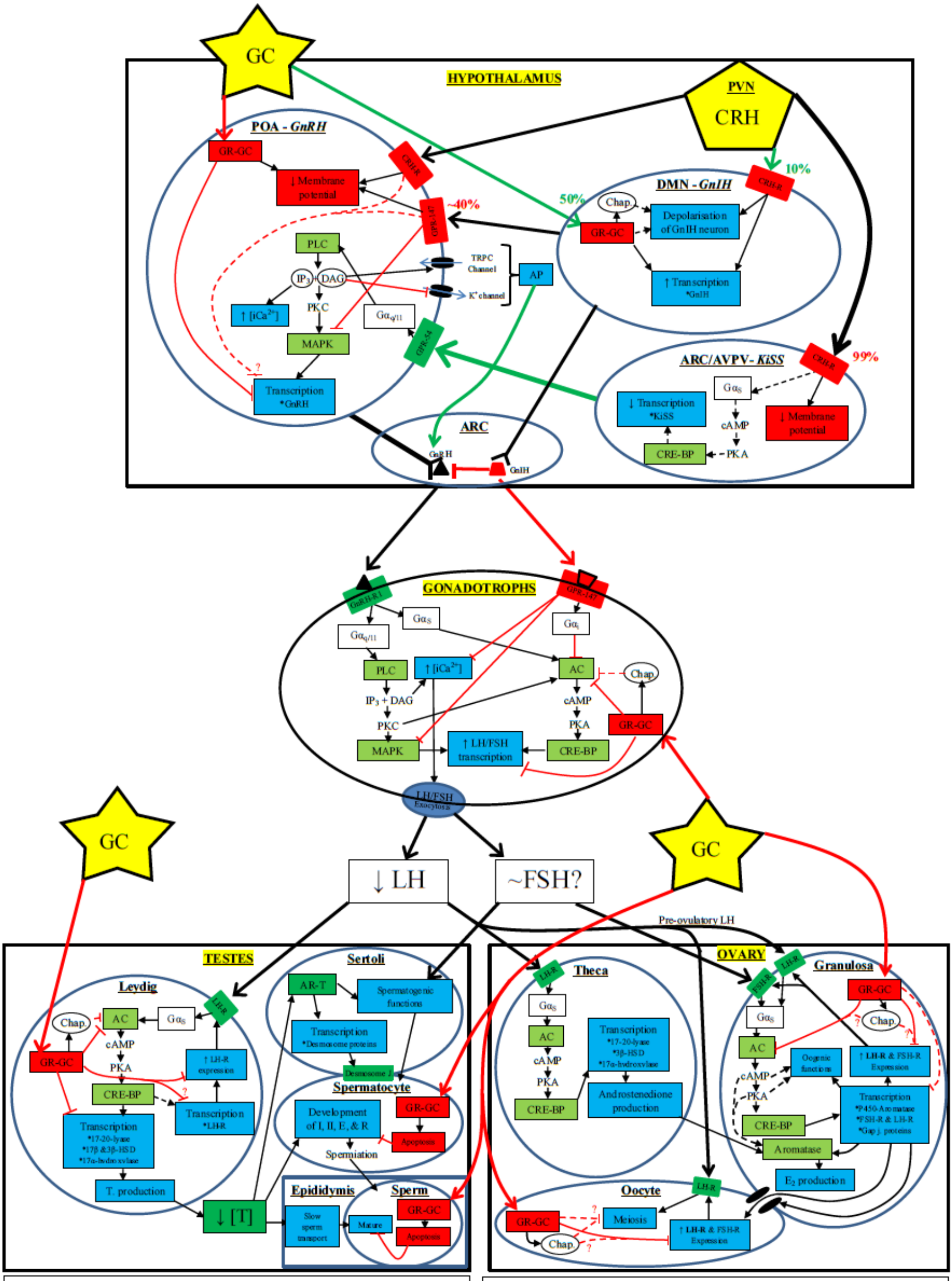

- Decrease plasma [testosterone]
- Disrupt sertoli-germ cell adhesion (↓ [T])
- Germ cell apoptosis (direct and via ↓ [T])
- Disrupt spermiation
- Slow epididymal sperm transport (via ↓ [T])
  - Incomplete development
  - High % abnormal sperm

- Decrease plasma [oestradiol] (via ↓LH/androstenedione &/OR ↓P450aromatase)
- ↓ Responsiveness to LH, and lesser extent, FSH (↓LH-R & FSH-R)
- Prevent/↓ preovulatory LH surge
- Disrupt or delay LH-surge-induced meiosis within oocyte