The Role of Dietary Isoflavones in the Reproductive and Hepatic Systems of Domestic and Non-domestic Feline Species

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This thesis is dedicated to the memory of "Angel", the cheetah whose illness initiated preliminary investigations into the potential link between dietary isoflavones and the health of captive cheetahs in 1987. Angel was a true ambassador for her species and her spirit will continue to live on in each new generation of cheetah ambassadors, as we continue to race against time in our efforts to save the cheetah from extinction.

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ABSTRACT

Dietary isoflavones are thought to influence reproductive and hepatic parameters in captive cheetahs. The isoflavone content of commercially-available feline diets was evaluated and isoflavones were found to be common constituents of diets consumed by captive cheetahs and domestic cats (occurring in over 75% of both diet types). Exposure of domestic cats was estimated to range between 0 and 8 mg/kg BW total isoflavones, whilst captive cheetah exposure was ranged from 0 to 4 mg/kg BW.

Single oral bolus doses of isoflavones were administered to captive cheetahs (n = 4) and domestic cats (n = 18) and serial blood, urine and faecal samples collected and analysed for isoflavone metabolite content. The fraction of isoflavone absorbed, as estimated from the plasma concentration over time, was 54% for genistein and 29% for daidzein in domestic cats. However cheetahs absorbed significantly less of both isoflavones (33% for genistein and 11% for daidzein). Sulphate conjugates predominanted the plasma metabolite profile (90% of plasma metabolites) in both species, but cheetah plasma contained approximately twice the amount of unbound genistein and daidzein than cats (as a fraction of the total detected). A dose- and/or diet-related response was observed in domestic cat studies but further testing is required to confirm this. Prior exposure to an isoflavone-containing diet appeared to enhance the production of equol, a metabolite of daidzein. The cheetah appears to be less efficient in its absorption of isoflavones, although this species is potentially inferior in its conjugation capacity. A positive correlation was observed between cheetah age and the proportion of absorbed fraction appearing as a conjugate in the plasma of this species.

Vaginal cytology was monitored in domestic cats consuming the purified isoflavones genistein and daidzein from weaning until 480 days of age and compared to that of unexposed, related cats. The reproductive tract from each cat was then removed during routine gonadectomy and a liver biopsy collected for comparison between groups. No difference in wet weight of reproductive tracts was detected. However, luminal epithelial cell height was greater in tissues from isoflavone-treated cats ($p < 0.05$). No differences were found in follicle development or distribution between groups and no histological abnormalities were detected. Expression of Oestrogen Receptor α and β was up-regulated in treatment cat tissues, while Progesterone Receptor expression was down-regulated, compared to control tissues ($p < 0.05$). Hepatic histology and the extent of fibrosis was unremarkable in both groups.

These findings indicate that despite their poor absorption and efficient conjugation, isoflavones consumed at doses representative of commercially-available diets are still capable of exerting biological activity in the reproductive tract of domestic cats. However no influence was detectable in the liver parameters measured. The potentially lower conjugation capacity of the cheetah may confer divergent biological activity in this species.

ABBREVIATIONS

4-HO-2-P	4-hydroxyphenyl-2-proprionic acid
ALP	Alkaline Phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
AUC	Area Under the Curve
BW	Body Weight
Cl	Clearance
CL	Corpora Lutea
C_{max}	Maximum concentration
CV	Coefficient of Variation
DHD	Dihydrodaidzein
DHG	Dihydrogenistein
DNA	Deoxyribose Nucleic Acid
DM	Dry Matter
E_1	Oestrone
E_2	Oestradiol
EGF	Epidermal Growth Factor
EH	Entero-hepatic
ER	Oestrogen Receptor
ERE	Oestrogen Response Element
EV	Extra-venous
FSH	Follicle Stimulating Hormone
GGT	Gamma Glutamyl Transferase
GIT	Gastrointestinal Tract
GnRH	Gonadotrophin Releasing Hormone
H	Kruskal-Wallis test statistic
h	Hour(s)
H & E	Haematoxylin and Eosin
HPLC	High Performance Liquid Chromatography
IGF	Insulin-like growth factor
IHC	Immunohistochemical

IU	International Unit
IV	Intra-venous
LCMS	Liquid chromatography mass spectrometry
LEH	Luminal Epithelial cell Height
LH	Luteinising Hormone
ME	Metabolisable Energy
min	Minute(s)
MOF	Multi-oocyte Follice
mRNA	Messenger Ribonucleic Acid
NBF	Neutral Buffered Formalin
O-DMA	*O*-desmethylangolensin
OVX	Ovariectomised
P_4	Progesterone
PCNA	Proliferating Cell Nuclear Antigen
PR	Progesterone Receptor
PGF	Prostaglandin
S/C	Sub-cutaneous
SEM	Standard error of the mean
SHBG	Sex Hormone Binding Globulin
SULT	Sulphotransferases
TGF	Transforming Growth Factor
T_{max}	Time of maximum concentration
TNF	Tumour Necrosis Factor
TRFIA	Time Resolved Fluro-Immuno Assay
UGT	UDP-glucuronosyltransferase
UV	Ultraviolet
Vd	Volume of distribution
VOD	Veno-Occlusive Disease

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Statement of Research Contribution

by Katherine Mary Bell

This thesis includes work which has been published in peer-reviewed, international journals. The work was conducted as part of the PhD candidature.

Section 2.1 was published as "Bell KM, Rutherfurd SM, Hendriks WH. (2006). The dietary isoflavone content of commercially available domestic cat diets in New Zealand. *New Zealand Veterinary Journal* 54(3): 103 – 108". Section 3.1 was published as "Bell KM, Pearce PD, Ugarte CE, Hendriks WH. (2006). Preliminary Investigation into the Absorption of Genistein and Daidzein by Domestic Cats (*Felis catus*). *Journal of Nutrition* 136: 2004S – 2006S".

Section 5.1 was published as "Bell KM, Ugarte CE, Tucker LA, Thomas DG. (2007). Genistein and daidzein do not affect puberty onset or oestrous cycle parameters in the domestic cat (*Felis catus*). *Asia Pacific Journal of Clinical Nutrition* 16(suppl. 3): S72." Results from Section 5.2 were published as "Bell K, Ugarte CE, Tucker LA, Roe WD, Thomas DG. (2008). Assessment of reproductive histology and sex steroid receptor expression in the domestic cat (*Felis catus*) following chronic exposure to phytoestrogens. *Reproduction in Domestic Animals* 43(Suppl. 3). Pp 126."

The candidate was the principal investigator for both studies and held the major responsibility for all aspects of these studies. The candidate designed, conducted, interpreted and wrote up all three studies. The candidate was responsible for the majority of sample collection analyses (dietary and biological samaple analysis by HPLC, vaginal cytology, immunohistochemistry, blood collection and pharmacokinetics, behavioural sampling, reproductive tract and ovarian gross histology and liver fibrosis quantification) and was responsible for all manuscript preparations. Input from co-authors was of an advisory, mentorship and critiquing nature.

Signed

D G Thomas, Chief Supervisor

CHAPTER ONE:

A Review of the Literature: Dietary Isoflavones and their Impact on Mammalian Reproduction and Hepatic Function

1.1. Introduction

The role that diet plays in mammalian health has received increasing attention in recent years. For example, there is evidence to suggest that the risk of certain hormone-dependent cancers and cardiovascular disease may be reduced in populations that include high amounts of soy in their diet. To this end, soy-derived phytoestrogens are generally thought to be responsible for these health promoting effects. However, the mammalian body is a complex series of interrelated systems, and what may result in benefit to one system may cause detrimental changes in another.

Cats have evolved as specialist carnivores with a variety of unique nutritional adaptations. Evolutionary pressure has selected digestive and metabolic processes that optimise such a carnivorous lifestyle, and as such many pathways utilised by plant-eaters to detoxify certain plant compounds have become redundant in the felids. Yet legumes such as soy are utilised as excellent sources of protein in the manufacture of pet foods for domestic cats. For this reason, attention has recently been drawn to the potential influence that soy-derived phytoestrogens may have in felid physiology.

Concern was first raised of the potentially detrimental impacts of dietary isoflavone consumption by felids after a study by Setchell *et al.* (1987ab) suggested a link between infertility in the captive cheetah and ingestion of relatively high doses of dietary soy. Although the work by Setchell *et al.* (1987ab) is frequently cited as having demonstrated an associative link in hepatic disease and speculative link to infertility, the study was not conclusive, due to its poor design and lack of control group. To date, no further work has been conducted to confirm their hypothesis. However, given their known intake of dietary soy isoflavones (Setchell *et al.* 1987ab; Court and Freeman 2004; Bell *et al.* 2006), the potential for isoflavone-induced reproductive perturbation still exists for felid species.

1.1.1 Phytoestrogen and Isoflavone Definition

Phytoestrogens are non-steroidal plant compounds with oestrogenic activity (Setchell 1995; Kurzer and Xu 1997; Santell *et al.* 1997; Setchell and Cassidy 1999). Three main classes of phytoestrogens exist; lignans, coumestans and flavonoids (which include flavones, flavanones, and isoflavones) (Kurzer and Xu 1997; Ginsburg and Prelevic 2000; Glazier and Bowman 2001). Genistein and daidzein have been identified as the major forms of isoflavones occurring in soy foods (Setchell 1995; Setchell 2000) (see Figure 1.1). However, there are a diverse range of soy-based foods available, and the isoflavone content of these foods varies dramatically (Andlauer *et al.* 2000a; Glazier and Bowman 2001; Setchell and Cole 2003).

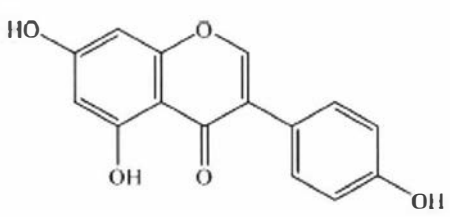
Interestingly, the biological reason for the presence of isoflavones in plants is uncertain. Some studies have postulated an anti-microbial role (Dixon and Ferreira, 2002), while others suggest that they may be involved in plant defence and root nodulation (Hur *et al.* 2000; Rafii *et al.* 2003).

1.1.2. Classification and Biochemistry

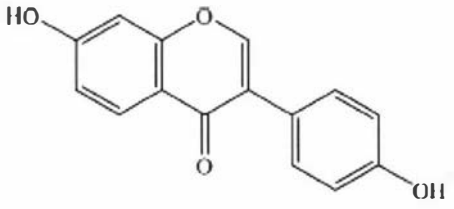
Isoflavones occur either as an aglycone (the parent form), or as a conjugated form with an attached glucose, glucuronic acid or sulphate moiety. Dietary forms include the "glycosides", "acetyl-glycosides" or "malonyl-glycosides", which are conjugated to a glucose moiety. Conjugates occurring as a consequence of *in vivo* metabolism include the "glucuronides", "sulphates" or "sulpho-glucuronides", depending on whether they are bound to a glucuronic acid, sulphate, or a combination of the two. These forms should not be confused with dietary forms, as different conjugating moieties may confer different biological activity. A distinction must also be made between ingested and endogenously derived aglycone forms since even though they may be chemically identical, their origin is relevant. By using the term "aglycones" strictly in reference to isoflavones administered to, or ingested by, an animal or person it is possible to

distinguish them from unbound circulating forms which have escaped enteric or hepatic conjugation, and/or have been deconjugated following absorption (discussed in Section 1.3). Hence, these circulating forms are termed "unbound" isoflavones.

All three classes of phytoestrogens are diphenolic, and some forms bear remarkable structural resemblance to both natural and artificial oestrogens and anti-oestrogens (Setchell and Cassidy 1999; Setchell 1995; Kurzer and Xu 1997; Ginsburg and Prelevic 2000; Glazier and Bowman 2001). With a diphenyl-propane structure where the B-ring is located at the 3-position (Murota *et al.* 2002), isoflavonoids can be divided into 12 isomers, including three main soybean isoflavones, found in any of four chemical forms (Kurzer and Xu 1997). Within the family of leguminous plants (including beans and peas) genistein is the simplest biosynthetic form (Dixon and Ferreira 2002).



Genistein (4',5,7-trihydroxyisoflavone),



Daidzein (4',7-dihydroxyisoflavone)

Figure 1.1. The molecular structure of the two major isoflavones, genistein and daidzein (from Brožič *et al.* 2006).

The predominant isoflavone forms occurring in soybean products are daidzin (4',7-dihydroxyisoflavone), genistin (4',5,7-dihydroxyisoflavone) and glycitin, which are the 6"-*O*-β-glycoside conjugate forms (Franke *et al.* 1994; Kurzer and Xu 1997; Coward *et al.* 1998; Setchell 2000; Setchell *et al.* 2001; Rafii *et al.* 2003). These isoflavones may also be found as an acetyl-glycosides and malonyl-glycosides (Kurzer and Xu 1997; Coward *et al.* 1998; Rafii *et al.* 2003). However, genistin and genistein are normally found in greater concentrations than daidzin and daidzein (Setchell *et al.* 2001). With

only a minor spelling adjustment to differentiate them, genistein and daidzein are the aglycone forms of genistin and daidzin, respectively.

1.1.2.1. *Processing effects*

During the processing of soy products, isoflavones are known to travel in association with the protein fraction (Setchell and Cassidy 1999). This processing is known to influence both the form and concentration of the isoflavones (Fukutake *et al.* 1996; Kurzer and Xu 1997; Coward *et al.* 1998), and significant losses have been reported after heat treatment (Eisen *et al.* 2003).

Unsurprisingly, foods which have undergone only minimal processing have isoflavone contents which are more reflective of raw soy beans than that observed in highly processed foods. For example, soy flour, which undergoes minimal processing, contains mainly the conjugates, 6"-O-malonyl-daidzin and 6"-O-malonyl-genistin (Kurzer and Xu 1997; Coward *et al.* 1998). However, heat treatment during extrusion processing (e.g. during the manufacturing of textured vegetable protein) causes the transformation of malonyl isoflavones to their respective acetyl forms (Kurzer and Xu 1997; Coward *et al.* 1998; Dixon and Ferraria 2002). Fermentive processing with bacterial cultures which are able to cleave the β-glycosyl bonds of isoflavone conjugates produces significantly greater proportions of aglycone forms than found prior to fermentation (Fukutake *et al.* 1996; Izumi *et al.* 2000; Hendrich and Murphy 2001; Setchell *et al.* 2002).

Processing can also reduce the overall isoflavone content. This is especially true when ethanol or aqueous extraction techniques are employed and the isoflavone-containing fraction is discarded (Hendrich and Murphy 2001). Unprocessed soybeans contain 1.2 – 4.2 mg isoflavone/g dry weight, whilst processed soy foods (e.g. textured vegetable protein) contain only 1.1 – 1.4 mg/g dry weight (Fukutake *et al.* 1996; Kurzer and Xu 1997; Setchell and Cassidy 1999).

1.1.3. Oestrogenic Activity

The oestrogenic activity of certain plants and isoflavones was noted as early as 1926 (Glazier and Bowman 2001). However, more comprehensive data did not become available until Bradbury and White (1954) listed 53 plants with oestrus-inducing capabilities, followed by a list of over 300 plant species with oestrogenic activity published by Farnsworth *et al.* (1975). Isoflavones are able to bind to oestrogen receptors (ER) and are termed natural selective oestrogen receptor modulators, as opposed to true oestrogens (Burke *et al.* 2000; Glazier and Bowman 2001; Nicholls *et al.* 2002; Setchell *et al.* 2002). The oestrogenic activity of isoflavones is discussed in more detail in Section 1.5.

1.2. Isoflavone Sources in Mammalian Diets

The association between soy and a variety of positive health effects, as well as its high quality protein source, have led to its increasing inclusion in the diets of a diverse array of mammalian species, including humans, poultry, swine, dogs and cats. Furthermore, dietary supplements containing isoflavones are also now available in human health food stores and marketed for their apparent health benefits.

1.2.1. Dietary Sources and Content

Soybeans were first shown to contain isoflavones in reasonably high concentrations in the 1930s (Walz 1931) and are perhaps the best known dietary source of genistein and daidzein. Since then assessment of the dietary isoflavone content of foods has become easier and more reliable with the recent validation of a number of databases (Mazur 1998; Hendrich and Murphy 2001; Kiely *et al.* 2003; USDA 1999; Ritchie *et al.* 2006). However, use of these databases may be limited to region and/or the plant variety from which the data was acquired. This is especially true in the case of soy-derived isoflavones since the isoflavone content of soybeans is known to be influenced dramatically by harvest time, genetic strain, geographical location of the crop, as well as year-to-year variation (Franke *et al.* 1994; Hendrich and Murphy 2001; Nakamura *et al.* 2001). Inter-batch variation as high as 300% has been reported for the soy protein content of soy protein isolates, and soy milk products may have 5-fold differences in isoflavone contents (Setchell and Cole 2003).

In addition to soybeans, other dietary sources of isoflavones include chick-peas, other legumes, clover, toothed medic and bluegrass (Kurzer and Xu 1997). The 4'methyl ethers of daidzein and genistein (formononetin and biochanin A, respectively) are found in clover (Kurzer and Xu 1997) while alfalfa is a common source of another phytoestrogen, coumestrol (USDA 1999). It follows that significant concentrations have been reported in non-soy containing animal diets. White *et al.* (2004) detected

isoflavones in corn gluten meal (32 μg/g daidzein and 50 μg/g genistein) and chicken meal (12 μg/g daidzein and 9 μg/g genistein), which were previously assumed to be phytoestrogen-free due to the lack of soy in their manufacture. The isoflavones found in chicken meal may occur as a result of the inclusion of chicken gastrointestinal contents, if chickens consumed soy-based poultry feed (White *et al.* 2004). Alternatively the tissue stores (e.g. the liver) of soy-exposed chickens may have contributed significant isoflavone concentrations. Yet the origin of isoflavones in corn gluten meal is yet to be explained. Nonetheless, finding isoflavones in non-soy ingredients indicates that soy's absence from a food's ingredient list cannot be used as a reliable indicator of low or negligible isoflavone content.

1.2.1.2. *Isoflavone content of feline diets*

Soybean and its fractions are utilised as ingredients in commercially prepared pet food (Court and Freeman 2002; Cerundolo *et al.* 2004) and diets intended for zoo-held carnivores (pers. obs.). The presence of genistein and daidzein in a commercial canine diet was first identified by Juniewicz *et al.* (1988), although quantification was not performed. Subsequently, only two scientific studies have investigated the isoflavone content of domestic canine or feline diets (Court and Freeman 2002; Cerundolo *et al.* 2004). These studies reported genistein contents of up to 163 and 559 μg/g dry matter (DM), and daidzein up to 147 and 615 μg/g DM, respectively. The isoflavone content of domestic and zoo felid diets has been assumed to result primarily from the inclusion of soy products in the formulation of these diets (Setchell *et al.* 1987ab; Court and Freeman 2002; Cerundolo *et al.* 2004).

On the basis of the dietary content (mg/kg DM) of isoflavones found in cat and dog foods (Court and Freeman 2002; Cerundol *et al.* 2004), it appears that isoflavone exposure to cats may be greater than that of dogs if intakes are similar on a DM per kg BW. Domestic cat diets typically have a higher protein content than diets intended for

domestic dogs, and this may explain their higher isoflavone content, if the protein fraction of feline diets is achieved through greater inclusion of soy protein.

Only one study has investigated the isoflavone content of a commercially-prepared diet fed to captive exotic felids (Setchell *et al.* 1987ab). This study identified genistein and daidzein (18 - 35 μg/g DM total) in a zoo carnivore diet, and reported its oestrogenic potential via a rodent bio-assay (Setchell *et al.* 1987a). Since no equine oestrogens were detected in the horse-meat based diet, the authors proposed that the source of the diet's oestrogenicity may have been soy isoflavones (Setchell *et al.* 1987b). The diet was reported to contain approximately 5 – 13% soy protein, and predicted to expose captive cheetahs to a daily dose of 50 mg total isoflavones. This was based on the consumption of 1 kg of the diet per day, and equates to a daily dose of 1.09 – 1.35 mg/kg body weight (BW) for an adult cheetah (calculated from a mean adult BW ranging from 37 – 46 kg; Marker and Dickman 2003). This finding may have far-reaching implications since the diet assessed (Setchell *et al.* 1987a) was one of the two most common diets reportedly fed to captive cheetahs in North America (Wildt *et al.* 1993).

1.3. Absorption and Metabolism of Dietary Isoflavones in Non-Felid Mammals

Confirmation of the presence of significant concentrations of isoflavones in feline diets does not automatically infer potential for biological activity. Once ingested, isoflavones need to cross the gut barrier before they can exert any systemic effect. Hence, the capacity of mammals to absorb and metabolise these compounds has direct relevance to their potential biological activity.

1.3.1. Absorption

Conjugated isoflavones do not typically cross the gut lining, whereas aglycones are absorbed directly (Izumi *et al.* 2000; Setchell 2000; Murota *et al.* 2002; Setchell *et al.* 2002). This is thought to result from the lipophobicity and greater molecular weight of conjugated isoflavones rendering them with lower affinity for the cellular membrane and less available for passive transport (Sfakianos *et al.* 1997; Andlauer *et al.* 2000ab; Hur *et al.* 2000; Setchell *et al.* 2001; Murato *et al.* 2002; Foti *et al.* 2006). Hence, the release of the aglycone forms (i.e. daidzein and genistein) from the conjugating moiety (Irvine *et al.* 1998ab; Setchell and Cassidy 1999) is critical to their absorption. The activities of glycosidase enzymes associated with the gut lining, and/or the gut microflora are known to be responsible for this key hydrolysis step.

Once within the enterocyte, aglycones are often re-conjugated (discussed in Section 1.3.2.2.) and these enteric conjugates may be transported primarily by specific, active transport processes, possibly within the brush border and basolateral membrane (Andlauer *et al.* 2000ab) (see Figure 1.2). However, no evidence of a specific transport mechanism for glucuronides was reported in the distal small intestine of rats (Sfakianos *et al.* 1997) and further research is necessary to elucidate the mechanism of isoflavone enteric transport.

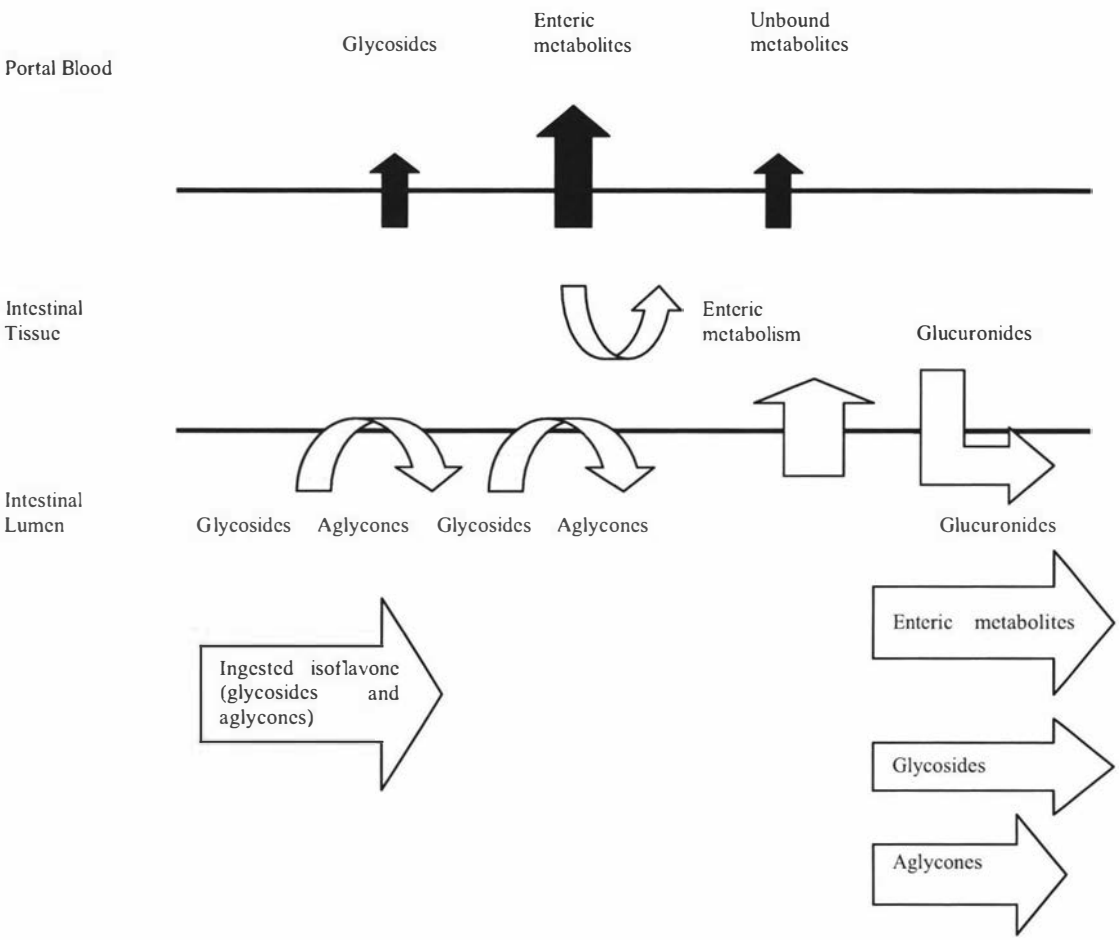


Figure 1.2. Schematic illustration of intestinal handling and metabolism of isoflavones. Adapted from Andlauer *et al.* (2000a).

Isoflavone-specific differences in enteric transport rate have been reported, with genistein passage known to be greater than daidzein due to the enteric formation of daidzein conjugates being rate-limiting (Chen *et al.* 2005b). Additionally, daidzein may have enhanced cellular uptake potential, thereby delaying its passage, since it has a greater affinity for the liposomal membrane than genistein (Murota *et al.* 2002). Enterocyte uptake accounts for 10% of the daidzein dose (Foti *et al.* 2006). This is greater than the equivalent proportion of uptake for genistein and may reflect differences in their structure and subsequent interaction with membrane phospholipids (Foti *et al.* 2006). These

differences may, in turn, result in divergent distributions to plasma and tissue compartments.

Interestingly, small portions of conjugated glycosides have been shown to cross the intestinal epithelial barrier (Andlauer *et al.* 2000a; Allred *et al.* 2005; Steensma *et al.* 2006). The detection of malonyl glycosides (found only in plants) in murine plasma following soy ingestion suggests that a fraction may cross the intestinal and hepatic barriers without modification (Allred *et al.* 2005).

The literature is divided in regards to the principal site of isoflavone absorption along the gastrointestinal tract (GIT). The colon is advocated by some studies (Sfakianos *et al.* 1997; Setchell *et al.* 2002; Foti *et al.* 2006; Cave *et al.* 2007a), whilst the upper intestine is considered more important in other studies (Setchell 2000; Turner *et al.* 2003). These apparent differences may be related to variations in GIT anatomy between species, or may simply reflect divergent analytical methodology.

1.3.1.1. *Gastrointestinal enzymes and microflora*

The numerous β-glycosidases along the mammalian GIT are thought to be important in isoflavone hydrolysis since they have a high affinity for isoflavones with a glucose residue at position 7 (i.e. most dietary isoflavones) (Izumi *et al.* 2000; Setchell *et al.* 2001; Setchell *et al.* 2002). Enzymes such as β-glucuronidase, β-glycosidase and lactase phloridzin hydrolase, are known to exist intracellularly, intracytosolically, and on the luminal side of the brush border membrane, respectively (Andlauer *et al.* 2000a; Turner *et al.* 2003; D'Alessandro *et al.* 2005). Hence, the mammalian GIT possesses the necessary enzymatic pathways to enable the absorption of dietary isoflavones. Phenol sulphokinases also occur in the intestine and liver, suggesting that sulphate conjugates entering the system (via biliary excretion, discussed in Section 1.3.2.1) could also undergo hydrolysis (Adlercreutz and Martin 1980).

In addition to GIT enzymes, intestinal bacteria such as *Lactobacilli*, *Bacteroides*, *Eischeria coli* and *Bifidobacteria* are also involved in isoflavone hydrolysis (Hur *et al.* 2000; Setchell 2000; Uehara *et al.* 2001; Setchell *et al.* 2002; Turner *et al.* 2003). Various strains of faecal bacteria possess hydrolytic and metabolic activity towards isoflavones, although differences were seen in metabolising capacity between bacterial strains, between isoflavones, as well as when bacteria were exposed as single species or mixed cultures (Hur *et al.* 2000; Ismail and Hayes 2005).

The presence of a malonyl- or acetyl- group at the C-6 position of the glucose ring has been linked to ionic or steric hindrance of enzyme activity (Ismail and Hayes 2005), and thus these dietary conjugates are likely to be absorbed poorly (Ismail and Hayes 2005). Additionally, the presence of genistein may exert some competitive inhibition over the hydrolysis of daidzein by faecal microflora (Ismail and Hayes 2005). These various factors are not often taken into account when comparing isoflavone absorption and metabolism and may explain differences in bioavailability estimates between studies. More importantly, the microflora status of individuals may vary and modulate isoflavone metabolism accordingly. The use of antibiotics or other causes of disruption to gut microflora have the potential to alter isoflavone metabolism and hydrolysis in the gut and must also be considered when assessing isoflavone absorption and subsequent bioavailability.

1.3.2. Metabolism

Following absorption, isoflavones may be metabolised into various forms. In the GIT isoflavones undergo various metabolic processes, including demethylation, hydroxylation, chlorination, iodination and nitration (D'Alessandro *et al.* 2005) (see Figure 1.3). Conjugation processes (phase II metabolism) occur in both the intestinal

wall and hepatic tissue, and involve the addition of a glucuronic acid or sulphate moiety to the isoflavone skeleton.

The conjugated isoflavones, bound to either glucuronic acid or sulphate, account for 85 – 99% of the total isoflavone content in humans, rats, mice and cats (Setchell *et al.* 2001; Uehara *et al.* 2001; Coldham *et al.* 2002b; Allred *et al.* 2005; Chang *et al.* 2000; Cave *et al.* 2007a). However, there is a large degree of variation among human subjects in the degree of isoflavone metabolite recovery (Kurzer and Xu 1997; Irvine *et al.* 1998b; De Boever *et al.* 2000; Glazier and Bowman 2001; Wiseman *et al.* 2004). Since the metabolites have unique and varying biological potential (see Section 1.4.4.6.), the differences in metabolic profiles observed between, and within species, is likely to result in variable responses to dietary isoflavone administration.

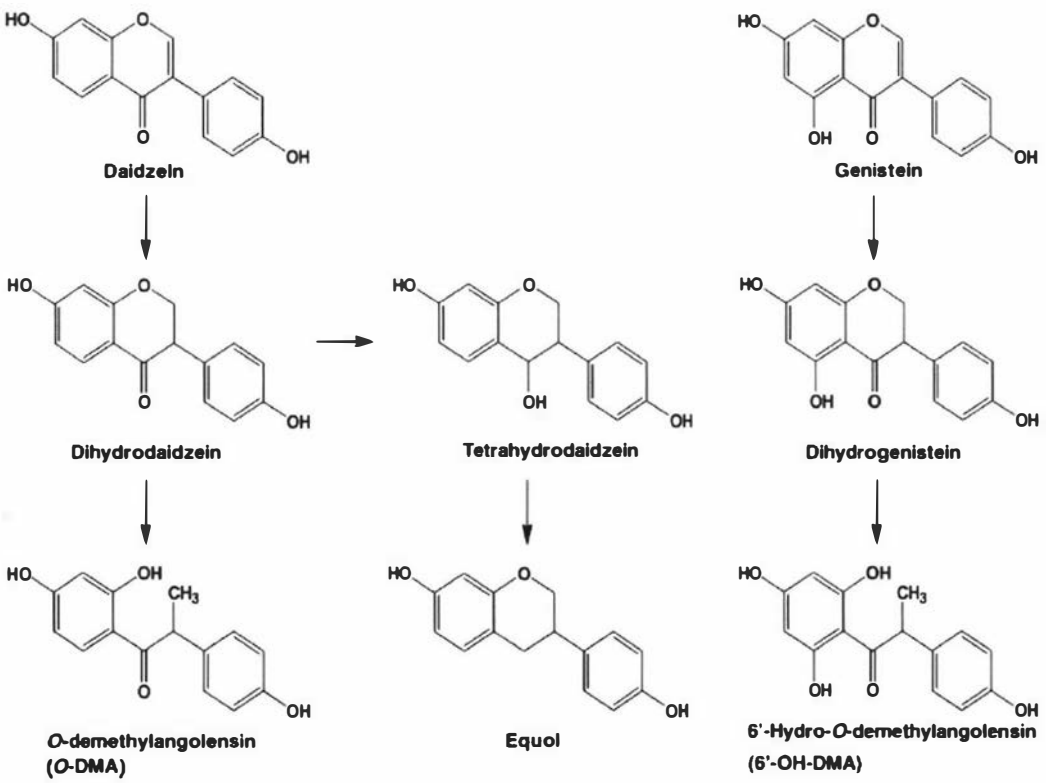


Figure 1.3. Metabolic conversion of daidzein and genistein. Following ingestion, isoflavones are cleaved by intestinal glycosidases to aglycones, and then further

metabolised by bacterial enzymes in the gut. Several metabolites are produced; dihydrogenistein, dihydrodaidzein, tetrahydrodaidzein, *O*-desmethylangolensin and equol (COT 2003).

1.3.2.1. *Hepatic metabolism*

Metabolism of isoflavones within the liver is reported to involve UDP-glucuronosyltransferases (UGTs), and sulphotransferases (SULTs), where glucuronic acid is thought to be the key conjugating moiety (Setchell 1995; Jäger *et al.* 1997; Kurzer and Xu 1997; Andlauer *et al.* 2000ab). Cytochrome P450 isoforms are also known to be catalytic in the hepatic metabolism and bioconversion of genistein and daidzein (Hu *et al.* 2003; Atherton *et al.* 2006).

Only trace amounts of unbound genistein have been detected in the bile, but both mono- and di-glucuronide, as well as sulphate metabolites are known to be present in greater concentrations (Jäger *et al.* 1997). The isoflavones occurring as glucuronides are thought to be preferentially maintained in the enterohepatic (EH) circulation (Setchell 1995; Setchell *et al.* 2001), but phase II metabolism is generally shown to be saturable (Sfakianos *et al.* 1997; Murato *et al.* 2002; Chen *et al.* 2003 and 2005b). Hence, a certain percentage of the absorbed unbound forms may enter circulation without further metabolism (Jäger *et al.* 1997; Sfakianos *et al.* 1997; Murato *et al.* 2002).

1.3.2.2. *Gastrointestinal metabolism*

It was previously thought that the liver was the only, or at least the principal, site of isoflavone conjugation, as is the case for endogenous steroids (Setchell *et al.* 2001). The GIT has indeed been found to possess a lower metabolic rate for isoflavones compared to the liver (Chen *et al.* 2005c), but orally ingested isoflavones appear at the intestine wall in concentrations greater than any other site of metabolism, purely due to its anatomical location. The finding that the majority of isoflavone forms found in portal venous blood

were glucuronides (Sfakianos *et al.* 1997; Setchell *et al.* 2001; Uehara *et al.* 2001; Murato *et al.* 2002; Chen *et al.* 2005c) indicated that conjugation occurred during passage through the enterocyte. Hence, the conjugating role of gastrointestinal tissue has now been acknowledged (Lundh 1990; Sfakianos *et al.* 1997) and the GIT is established as the tissue primarily responsible for first-pass metabolism of isoflavones (Setchell *et al.* 2001).

Gastrointestinal metabolism was further illustrated by Prasain *et al.* (2006), who reported that genistin β-glycoside infused directly into the femoral or portal vein, remained unchanged as it passed through the body and liver, and appeared intact in the urine and bile. However, infusion of the glycoside into the small intestine resulted only in the 7β-*O*-glucuronide of genistein being present in the bile, with no detectable glycoside present (Prasain *et al.* 2006).

As is known with hepatic metabolism, a saturation threshold is thought to exist for the enteric conjugating capacity. This may be a consequence of the down regulation or inhibition of UGT enzymes, or a greater affinity of the unbound form for carrier proteins (Foti *et al.* 2006). The implication of a saturable metabolic capacity is that bioavailability (and hence biological activity) of isoflavones may not exhibit a predictable dose-related response. The conjugation process is thought to enhance excretion, and potentially reduce biological activity (discussed below in Section 1.4.4.6), so saturation of this process at high doses may result in a greater proportion of isoflavones entering circulation in their unbound forms than observed at lower doses. The greater presence of these more biologically active forms may result in physiological responses that exceed that predicted from a linear dose-response.

Glucuronide conjugates are the predominant forms of metabolite detected for daidzein or genistein in rat intestinal perfusion trials, whereas sulphate, disulphate or sulphoglucuronide conjugates are not generally detected (Sfakianos *et al.* 1997; Andlauer

et al. 2000a). Since sulphate metabolites have been detected in circulation after oral isoflavone exposure, the addition of sulphate moieties to isoflavone skeletons is thought to be a function limited to the liver (Adlercreutz and Martin 1980). This idea is supported by more recent work investigating rat liver sulphotransferase activity which was found to be 4-fold higher than intestinal activity for genistein, although it was equivalent for daidzein sulphation (Ronis *et al.* 2006b). However, in humans, genistein and daidzein sulphation occurs throughout the GIT, with intestinal sulphotransferase activity greater than that of hepatic SULTs (Chen *et al.* 2005b; Ronis *et al.* 2006b). Interspecific differences between canine and human intestinal sulphation is also reported by Adlercreutz and Martin (1980). The consequence of these species-specific differences in metabolism may include variable biological responses to dietary isoflavones since sulphate metabolites may confer alternative biological effects to glucuronide conjugates.

Genistein is reportedly glucuronidated faster than daidzein throughout the GIT, and also to reach saturation at a higher concentration in the jejunum (Chen *et al.* 2005c). This may be due to the existence of the two electron-donating groups on the A-ring of genistein making it more available for conjugation (Chen *et al.* 2005c). However, the effect is unlikely to persist in the liver since hepatic microsomes are more complex, whereby the 7-hydroxyl position appears to be more important for glucuronidation in this tissue (Chen *et al.* 2005c) and genistein would not possess any structural advantage over daidzein. Therefore, in species with functional hepatic metabolism the two isoflavones would not be predicted to differ in the extent of glucuronidation. On the other hand, species with only limited hepatic metabolism may exhibit greater concentrations of genistein glucuronides than daidzein glucuronides.

The intestinal handling and metabolism of isoflavones is schematically represented in Figure 1.2, based on the findings reported in the literature. The role of the GIT tissue in isoflavone metabolism has an important bearing on the relevance of studies administering isoflavones via injection, effectively by-passing GIT metabolism.

The crucial role for intestinal microflora in isoflavone metabolism was demonstrated by the decrease in equol and *O*-desmethylangolensin (*O*-DMA) production observed both during antibiotic therapy, and in germ-free rats (Atkinson *et al.* 2005; Cassidy 2005). However, the oral intake of commensal microflora through the use of probiotics (e.g. *Lactobacillus acidophilus* and *Bifidobacterium longum*) has been clearly demonstrated to be ineffective in modulating isoflavone metabolism and does not influence equol or *O*-DMA production (Bonorden *et al.* 2004; Clavel *et al.* 2005; McMullen *et al.* 2006) or genistein or daidzein excretion (McMullen *et al.* 2006). Hence, although these micro-organisms are known to be important factors in isoflavone absorption and metabolism, some interaction with other endogenous factors must be involved.

1.3.2.3. *Genistein metabolites*

Genistein metabolism is known to involve its conversion to dihydrogenistein (DHG), before further metabolism to 6'-hydroxy-*O*-DMA (Cimino *et al.* 1999; Coldham *et al.* 1999 and 2002a; Joannou *et al.* 1995), or 4-hydroxyphenyl-2-propionic acid (4-HO-2-P) (Coldham *et al.* 1999 and 2002a) (see Figure 1.3). Reduction and heterocyclic ring fission produces 4-HO-2-P, whilst further metabolism to *p*-ethyl-phenol is possible by decarboxylation (King 1998; Coldham *et al.* 1999; Coldham and Sauer 2000; De Boever *et al.* 2000). However the presence of *p*-ethyl-phenol is not consistently reported. This may be due to interspecific differences or intraspecific variation owing to dietary, analytical sensitivity or gut microflora differences (Coldham and Sauer 2000).

The caecum is thought to be the initial site of bacterial genistein metabolism in rats (Coldham *et al.* 1999 and 2002a). Further metabolism of genistein has also been reported to occur in the enterocytes and liver, whereupon the majority of orally ingested genistein was conjugated by phase II metabolising enzymes (Setchell 1995 and 2000; Busby *et al.* 2002; Dixon and Ferraria 2002; Doerge *et al.* 2002; Saitoh *et al.* 2004). During intestinal transit, glucuronic acid is added to the genistein skeleton, primarily at the 7-hydroxyl group, to form the 7-*O*-β-glucuronide, although conjugation at the 4 and 4' position has

also been reported (Sfakianos *et al.* 1997; Coldham and Sauer 2000; Doerge *et al.* 2000; Setchell 2000). Plasma glucuronides of genistein make up the majority of circulating genistein.

1.3.2.4. *Daidzein metabolites*

Daidzein is metabolised to dihydrodaidzein (DHD), before being converted to equol and/or *O*-desmethylangolensin (*O*-DMA) by intestinal microflora (Joannou *et al.* 1995; Setchell 1995; Cimino *et al.* 1999; Heinonen *et al.* 1999; Setchell *et al.* 2001; Rafii *et al.* 2003) (see Figure 1.3). Other intermediate metabolites are also known to exist, including tetrahydrodaidzein, 4-OH-equol and 2-dehydro-*O*-DMA (Joannou *et al.* 1995; Heinonen *et al.* 1999).

Since equol and *O*-DMA do not generally appear in plasma or urine until approximately 6 – 8 h following ingestion, with peaks occurring much later (around 24 h), these metabolites are thought to be produced and absorbed in the colon (Setchell 1995; King *et al.* 1996; King 1998; King and Bursill 1998; Setchell *et al.* 2001; Setchell *et al.* 2003a). However, reports that equol and *O*-DMA appeared in greater concentrations in urine than in faeces (4.0 *versus* 1.9% for *O*-DMA or 7.0 *versus* 1.6% for equol, respectively) (Watanabe *et al.* 1998) suggest that equol is either efficiently absorbed from the colon, or that some post-absorption conversion of daidzein is also possible.

Studies suggest that only between 33% and 44% of the human population excrete significant quantities of equol after isoflavone consumption (Lampe *et al.* 1998; Lu and Anderson 1998; Slavin *et al.* 1998; Setchell *et al.* 2003ab; Cassidy *et al.* 2006; Mathey *et al.* 2006). This may result from inherent metabolic variations, differences in microflora, unmonitored metabolite destinations, broad tissue distribution and/or processing techniques (Setchell 1995; Kurzer and Xu 1997; Setchell 2000; Setchell *et al.* 2001). However, equol production is not thought to be related to gender (Cassidy *et al.* 2006).

Domestic dogs have also been found to be capable of converting isoflavones found in commercial pet food to equol (Juniewicz *et al.* 1988), although quantification of the dose ingested and fraction excreted as equol was not reported.

Circulating forms of daidzein are predominantly conjugated (Doerge *et al.* 2000; Setchell 2000; Busby *et al.* 2002; Karakaya *et al.* 2004; Saitoh *et al.* 2004; Allred *et al.* 2005). Importantly, the circulating concentrations of unbound equol in humans is known to be higher than its parent compound, daidzein (49.7% *versus* 18.7%, respectively), and equol also has a longer retention time (Rafii *et al.* 2003). As such, the biological potential of daidzein may be underestimated if its metabolites are not taken into consideration since equol is known to possess greater ER binding affinity than daidzein (discussed in Section 1.4.4.6). Additionally, interspecific differences in the proportion of daidzein and its metabolites occurring as glucuronides, sulphates or in the unbound form are commonly reported in the literature (Doerge *et al.* 2000; Piskula 2000; Setchell 2000; Allred *et al.* 2005; Karakaya *et al.* 2004). This has important implications for the comparison of potential biological activity of daidzein between species.

1.3.3. Excretion

Following absorption, a certain fraction of the isoflavone dose is known to be excreted from the body via the urine or faeces (see Figure 1.4). The majority of the excreted isoflavone fraction is typically accounted for within the 24 - 48 h period following exposure (Coldham and Sauer 2000; Setchell 2000). The proportion of isoflavone dose appearing in the urine and faeces appears to vary between studies, species and isoflavone.

1.3.3.1. *Enterohepatic recirculation*

Isoflavones, and genistein in particular, undergo enterohepatic (EH) circulation, whereby they are extracted by the liver from portal blood and transported into the bile (Adlercreutz

and Martin 1980; Sfakianos *et al.* 1997; Setchell 2000; Coldham *et al.* 2002b). Biliary secretion is also an important elimination pathway of sex steroids in most mammals (Shille *et al.* 1984; Schwarzenberger *et al.* 1996; Brown *et al.* 1994; Brown *et al.* 1996; Brown *et al.* 2001) and the structural resemblance of genistein to oestradiol may predispose it to this excretory pathway.

The biliary metabolites may either be deconjugated repeatedly by gut bacteria and subsequently reabsorbed, or may undergo additional metabolism and/or degradation in the distal intestine (Kurzer and Xu 1997; Sfakianos *et al.* 1997; Setchell *et al.* 2001; Coldham *et al.* 2002b; Thomson 2005). Biliary metabolites may therefore be retained in circulation or be excreted in the faeces. As such, the faecal isoflavone content may represent isoflavones that have passed directly through the digestive system without absorption into circulation, and/or isoflavones that have undergone EH recirculation. The pathway taken by isoflavones is imperative to the understanding of their potential biological activity but cannot easily be determined.

Enterohepatic recirculation also effectively slows the time taken for isoflavones to be cleared from the body, and as much as 75% of an infused genistein dose was reported to be sequestered in the bile (Sfakianos *et al.* 1997). Hence, the same isoflavone molecule may appear twice within the plasma profile and artificially inflate the estimated proportion of dose that is absorbed. Indeed, the presence of a second peak in the plasma appearance curve has been used to demonstrate enterohepatic recirculation *in vivo* (Coldham and Sauer 2000; Setchell 2000; Setchell *et al.* 2001; Coldham *et al.* 2002b; Steensma *et al.* 2006). Yet this second peak may affect the calculation of the area under the curve, which is then used to estimate the proportion of ingested dose appearing in the plasma.

Other implications of an effective EH circulation include the build up of body isoflavone pools, and a longer half life than previously assumed (Sfakianos *et al.* 1997; Zhang *et al.*

1999; Thomson 2005). Portal vein and hepatic exposure to isoflavones is likely to be significantly underestimated by peripheral concentrations (Uehara *et al.* 2001) and localised activity in these tissues should be considered.

Importantly, hepatobiliary transport of genistein is known to involve saturable steps, with biliary excretion concentrations reaching saturation point at higher intestinal infusion dosages (Sfakianos *et al.* 1997), which may contribute to the non-linear plasma kinetics observed. Ultimately this will play a role in an isoflavone's biological activity at high doses as any proportion of the dose escaping EH recirculation may be released into circulation, modulating the temporal exposure of an animal.

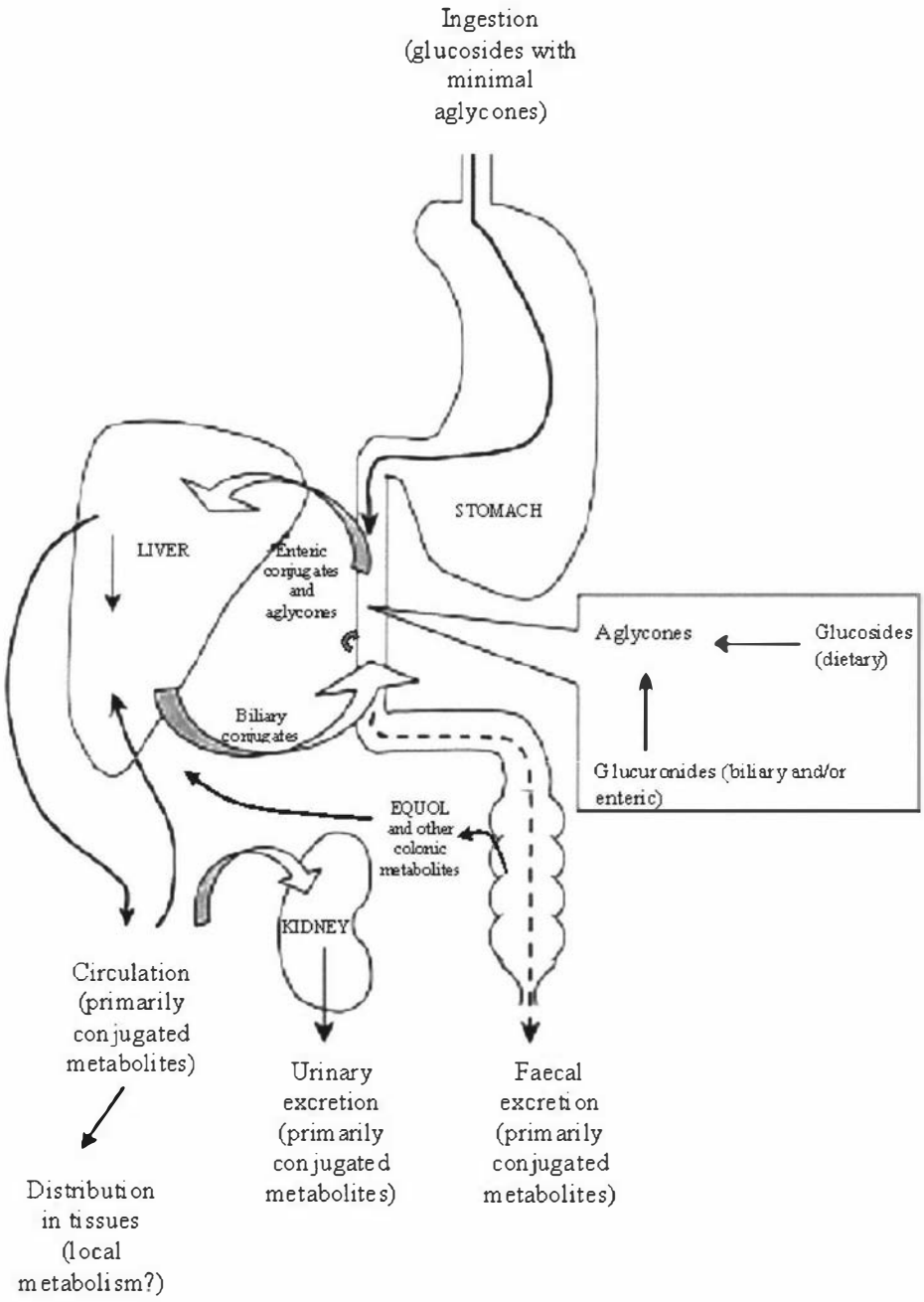


Figure 1.4. Schematic illustration of the physiological fate of dietary isoflavones (adapted and extended from the illustration of Turner *et al.* 2003; Thomson 2005).

1.3.3.2. *Urine*

Isoflavones found in the urine must have been absorbed prior to excretion and therefore provide a rudimentary indication of absorption. However, caution is needed when

interpreting urinary data due to the varying preferences for the renal excretory route between genistein and daidzein. Isoflavones are excreted in urine predominantly as conjugates (Lu *et al.* 1995; Irvine *et al.* 1998b; Lu and Anderson 1998; Zhang *et al.* 2003), with most excretion occurring within 12 – 24 h of isoflavone exposure (Xu *et al.* 1994; King *et al.* 1996; King and Bursill 1998; Lu and Anderson 1998; Zhang *et al.* 1999; Zhang *et al.* 2001; Busby *et al.* 2002). Estimates of urinary recovery range from 21 - 63% for daidzein, and 0.5 - 67% for genistein, with daidzein excretion consistently greater than that of genistein within each study (Zhang *et al.* 1999; Coldham and Sauer 2000; Shelnutt *et al.* 2000; Zhang *et al.* 2001; Bloedon *et al.* 2002; Busby *et al.* 2002; Zhang *et al.* 2003; Tsangalis *et al.* 2005).

1.3.3.3. *Faeces*

Detecting isoflavones in the faeces can mean one of two things. Either these isoflavones passed straight through the animal's gut, or were absorbed and subsequently secreted back into the gut lumen. The natural sloughing of enterocytes, potentially containing partially absorbed or metabolised isoflavones may also contribute to faecal isoflavone recovery, whereas biliary isoflavones involved in EH circulation may further increase faecal isoflavone concentrations.

Some studies have suggested that faecal excretion of isoflavones by humans is minimal (Xu *et al.* 1994 and 1995; Setchell *et al.* 2003b). However, reports of only 0.4 – 4.4% of ingested dose of either genistein or daidzein in human faeces (Xu *et al.* 1994 and 1995; King 1998; Watanabe *et al.* 1998) contrast with the 20 – 35% recovery of genistein in rat faeces (King *et al.* 1996; Steensma *et al.* 2006) and support the notion of interspecific variation in faecal isoflavone excretion (Yoshida *et al.* 1985). However, another study reported 2.3% and 3.4% of the daidzein and genistein dose (respectively) in rat faeces (King 1998), indicating that differences may be methodological rather than species-specific.

1.3.4. Pharmacokinetics

The manner in which isoflavones behave in the mammalian body following absorption may be best described using pharmacokinetic analysis. This process describes the plasma appearance and disappearance of isoflavones, and permits the calculation of the duration of exposure, circulating plasma concentrations over time, and can be used to predict the distribution of isoflavones to non-plasma body compartments. However, a number of flaws in study design can lead to the misinterpretation of pharmacokinetic data and erroneous conclusions may be drawn regarding isoflavone bioavailability.

For example, the majority of published studies utilise enzymatic or acid hydrolysis of blood, urine or faecal samples prior to the assessment of isoflavone content. As such they report the "total" isoflavone content as equivalents in their unbound form. This represents the sum of all metabolites and naturally occurring unbound forms, which, as a single value, is unlikely to represent the behaviour of each individual metabolite (Bloedon *et al*. 2002; Busby *et al*. 2002).

Furthermore, the number of samples taken following isoflavone exposure is important when assessing any pharmacokinetic parameter. It is doubtful that an accurate reflection of bioavailability, area under the curve (AUC), or time of peak concentration (T_{max}), could be obtained from only a few time points sampled within the first eight hours following exposure. Studies utilising such a limited sampling regime may also erroneously report differences between unbound and conjugated forms, or between genistein and daidzein. Additionally, failure to sample sufficiently frequently during the terminal elimination phase may significantly underestimate the terminal half life and AUC of isoflavones (Cave *et al.* 2007a), which has important consequences when predicting the likely duration of exposure and possibility for systemic effects.

	Genistein	Daidzein	Comments
Time to reach maximum plasma concentration (T_{max})	0.5 – 9.3 h	1.17 – 9.0 h	Rats appear to have shorter T_{max} values, possibly due to gut transit time, dose formulation, prior fasting or sampling schedule.
Maximum plasma concentration (C_{max})	3440 – 28158 nmol/L (total). 47 – 360 nmol/L (unbound)	1719 – 8973 nmol/L (total). 21 – 99 nmol/L (unbound)	Dose-responsive. Linearity not consistently reported. Genistein typically greater than daidzein
Half life	5.74 h – 12.90 h	4.71 h – 16.00 h	Likely to be influenced by dietary matrix, dose, feeding duration, sampling duration as well as the forms fed and measured. Reduced half life of conjugates may be due to increased excretion whilst greater distribution of unbound forms will extend their half life.
Area under the curve (AUC)	4540 ng h/ml (total). 153 – 257 ng h/ml (unbound). 1234 – 5667 ng h/ml (conjugated).	2940 ng h/ml (total).	Generally dose-responsive although curvilinear relationship with dose suggests saturability of absorption/excretion.
Volume of distribution (V_d)	44 – 66 L/kg (unbound). 1.33 – 2.12 L/kg (total).	81 – 245 L/kg (unbound). 1.41 – 3.28 L/kg (total).	Daidzein typically has a greater V_d than genistein although conflicting reports exist. Unbound fractions have greater V_d than conjugated fraction indicating greater tissue sequestering of these forms
Clearance rate (Cl)	21.85 L/h	30.09 L/h	Daidzein clearance is reflective of its greater V_d.

Table 1.1. Pharmacokinetic parameters compiled from the literature in humans and rats (King and Bursill 1998; Watanabe *et al.* 1998; Setchell and Cassidy 1999; Coldham and Sauer 2000; Izumi *et al.* 2000; Setchell 2000; Setchell *et al.* 2001; Uehara *et al.* 2001; Busby *et al.* 2002; Bloedon *et al.* 2002; Coldham *et al.* 2002b; Howes *et al.* 2002; Richelle *et al.* 2002; Shelnutt *et al.* 2002; Setchell *et al.* 2003ab; McClain *et al.* 2005; Soucy *et al.* 2006).

In studies involving multiple sampling points, the initial part of the plasma appearance curve is most likely a reflection of the absorption process. However, beyond the peak plasma concentration a combination of absorption, metabolism, distribution, excretion and entero-hepatic recirculation is likely to be occurring. This makes it difficult to distinguish a true elimination rate.

Considerable variation exists in the pharmacokinetic parameters reported for humans and rats (see Table 1.1) and it is unlikely that either species would make an appropriate comparison or model for felids. Most of this variation is thought to be due to variable methodology, dose, dietary matrix, and form of isoflavone administered, as well as anatomical and physiological differences. Furthermore, elimination from the plasma compartment does not necessarily reflect systemic elimination, so the dynamic flux of isoflavones between various body tissues and/or their further metabolism, must also be taken into account. The storage of isoflavones in body tissues and their gradual or periodic release back into circulation makes interpretation of plasma data difficult.

1.3.5. Bioavailability

1.3.5.1. *Definition and assessment*

The term "bioavailability" is subject to variable definitions, depending on the field of expertise (Wienk *et al.* 1999). Indeed, according to the European Medicines Evaluation Agency (human guidelines) bioavailability is defined as "the rate and extent to which the active substance or moiety is absorbed from a pharmaceutical form, and becomes available at the site of action" (Anonymous 2001). Measurement of the absolute bioavailability requires that the plasma AUC following extra-venous (E/V) (i.e. *per os*) administration is expressed as a proportion of the AUC obtained following intra-venous (I/V) administration of the same compound, at an equivalent dose (Toutain and Bousquet-Mélou 2004). However, I/V administration is rarely included in nutritional studies (exceptions being Coldham *et al.* 2002b and Cave *et al.* 2007a). Bioavailability in this field has hence taken on the broader definition of the "efficiency with which nutrients are utilised", and is measured as the "proportion of the total [nutrient/compound] in a food or diet that is digested, absorbed and metabolised by normal pathways" (Wienk *et al.* 1999).

There are a number of limitations to the estimation of bioavailability. Any serial sampling of blood concentrations is limited by the fact that a total collection of plasma is not possible and therefore the risk of missing peaks in concentration is inherent. Additionally, due to the complex process of isoflavone transport in the body, including the re-circulation of isoflavones in the enterohepatic system and/or through cellular uptake and secretion, it is possible that a single isoflavone molecule may be detected twice, or even multiple times, along a single plasma concentration-time curve. Calculations of the fraction of dose absorbed may be overestimated in these situations.

A further complexity is incorporated from the large number of possible end-points for isoflavones in the body following absorption, which may represent long-term sites of sequestration or simply transitional stores. Biliary secretion may result in re-absorption or excretion in the faeces and all potential sinks or body components that isoflavones pass through have the potential to become sites of degradation or metabolism of isoflavones. Hence, a comparative or absolute estimate of genistein or daidzein bioavailability may be best drawn from studies identifying and screening for all known metabolites, in which duplicate measurements are avoided through the simultaneous screening of end-points. Using a mass balance study design and/or radio isotope labelling may better describe the pharmacodynamics of isoflavones within the body.

The use of urinary excretion in the calculation of bioavailability assumes that renal clearance is measurable, variable among test subjects and that the isoflavone exhibits 100% clearance via the kidneys (Setchell *et al.* 2003b; Toutain and Bousquet-Mélou 2004). The last of these assumptions cannot be met for isoflavones, especially genistein (Sfakianos *et al.* 1997), and has led to the erroneous reporting of lower genistein bioavailability compared to daidzein by a number of authors (e.g. Xu *et al.* 1994; Irvine *et al.* 1998b; King 1998). Moreover, once excreted in the urine, an isoflavone is no longer available to systemic circulation for potential biological activity. Therefore an isoflavone with high urinary recovery may actually be considered less bioavailable, relative to an isoflavone with poor urinary recovery, which has increased systemic

retention. Measurement of isoflavone recovery in all physiological compartments would be necessary to test this hypothesis.

1.3.5.2. *Isoflavone bioavailability*

Isoflavone bioavailability is poor in all species studied to date. Having said that, physiological changes have still been associated with the consumption of isoflavones from a dietary intake of soy and its products, which include lower doses than often used in clinical trials (see Section 1.5.2). Hence, the dose actually absorbed and available for activity is less than the minimum effective oral dose required to produce physiological changes.

Bioavailability estimates for the unbound form of genistein range from 6.8 - 14.6% of the ingested dose in rats (Coldham *et al.* 2002b), to 1.38 - 29.85% for the unbound and conjugated forms, respectively, in cats (Cave *et al.* 2007a). Gender and species-specific differences in bioavailability are reported, and the use of different methods to estimate bioavailability further complicates interpretation of data. Overall, the low estimates of bioavailability are thought to occur due to poor absorption (approximately 28% of perfused aglycones were absorbed in a rat intestinal perfusion study, Chen *et al.* 2003), the inclusion of isoflavones in enterohepatic and enteric recirculation, and their efficient metabolism and excretion (Chen *et al.* 2003; Cave *et al.* 2007a). This is further supported by findings which support the existence of a saturable, rate-limiting uptake mechanism, including nonlinear bioavailability estimates (Setchell *et al.* 2003b).

The low bioavailability of isoflavones adds a curious intricacy to the discussion of their biological potency. The high oral doses required to achieve minimal biological changes in some studies (e.g. McClain *et al.* 2005 and 2006b) may simply reflect the efficient bioconversion and/or excretion of isoflavones under those study conditions, rather than their low potency. Yet, ultimately the biological consequence would be the same for a

compound with high biological potency but poor bioavailability, compared to one with low biological potency but high bioavailability. This emphasises the importance of determining the bioavailability of a compound when assessing biological activity and highlights the need to recognise the irrelevance of studies utilising routes of administration which do not include intestinal transit.

1.3.6. Isoflavone Tissue Distribution

Bioavailability estimates are based on the concentration of isoflavones occurring in plasma, urine and/or faecal compartments. However, since isoflavone activity may occur at the cellular or tissue level it is important to also consider the distribution and concentration of isoflavones in tissues.

Genistein and daidzein are known to be widely distributed in the tissues of the body, although primary target tissue concentrations vary among species (Setchell *et al.* 2001). Target organs for isoflavone accumulation include the liver, reproductive tract, GIT and kidney, whereas concentrations are generally lower in the skeletal muscle, spleen and brain (Yueh and Chu 1977; Coldham and Sauer 2000; Tsai 2005). It could therefore be predicted that isoflavone activity may be more apparent in these former tissue types.

Interestingly, the adipose tissue is reported to be poorly perfused by isoflavones (Coldham and Sauer 2000; Schlosser *et al.* 2006). This may be due to the predominance of isoflavones circulating as polar, lipophobic metabolites. Situations resulting in higher concentrations of lipophilic, unbound isoflavones may be associated with greater fat storage and a potentially longer residence time in the body. However, these differences would not be predicted from studies reporting only total unbound equivalents, and underlines the importance of determining the circulating metabolite profile.

It follows that concentrations of unbound forms in tissues typically exceed that reported in plasma, and make up a greater fraction of the total tissue isoflavone body content (10 – 100%), compared to the respective fraction found in the plasma (1 – 5%) (Chang *et al.* 2000). Of particular importance is the unbound concentration found in target tissues, relative to the concentration known to elicit biological activity. In this regards, plasma concentrations and metabolite profiles do not appear to be appropriate indicators of biological potential, and the results from *in vitro* studies using doses equivalent to reported plasma concentrations may significantly underestimate exposure within tissues *in vivo*.

1.3.6.1. *Intestinal tissue isoflavone content*

Storage of isoflavones in intestinal tissue was reported to be high following oral ingestion, whereby the GIT content represented the highest concentration of genistein compared to other body organs (Coldham and Sauer 2000). Within the GIT, the small intestine and caecum exhibited the highest concentration (Coldham and Sauer 2000). Isoflavone accumulation in the intestine may reduce systemic availability of isoflavones and, in particular, may reduce the exposure of target organs such as the reproductive and hepatic tissues. However, localised activity within the intestinal tissue may also occur.

1.3.6.2. *Hepatic isoflavone concentration*

The liver is the central processing organ for compounds entering the body via the GIT and is the focus of many toxicological investigations. Prior to release into peripheral circulation, isoflavones may be retained in the liver and exert localised effects on the hepatic tissue. Following a single oral dose, the liver concentration of genistein in rodents was among the highest of all body organs (Chang *et al.* 2000; Coldham and Sauer 2000), with 77 - 100% of the hepatic genistein content occurring in the unbound form (see Table 1.2) (Chang *et al.* 2000; Gu *et al.* 2005; Chen and Bakhiet 2006; McClain *et al.* 2006b). These liver concentrations occurred in the presence of significantly lower

circulating serum concentrations, of which only 0 – 2% was present in the unbound form (Chang *et al.* 2000; Tsai 2005; McClain *et al.* 2006b). These findings indicate that activity may be significantly greater in the liver than predicted from circulating concentrations.

Interspecific differences have been observed in hepatic isoflavone storage in rats and dogs following long-term exposure to genistein (McClain *et al.* 2005; McClain *et al.* 2006b). Although the small sample size in the dog study makes interpretation difficult, overall dogs appeared to store lower proportions of ingested genistein in the liver, with a smaller relative proportion of the unbound form, ranging from 16 – 84% (see Table 1.2), compared to rats. Gender-specific differences were also observed, whereby female rats and dogs typically exhibited greater genistein contents than males in equivalent treatment groups (Chang *et al.* 2000; Coldham and Sauer 2000; McClain *et al.* 2005; McClain *et al.* 2006b). Additionally, predominantly sulphated genistein was found in male livers but female livers contained mainly unbound genistein (Chang *et al.* 2000; Coldham and Sauer 2000). A number of potential mechanisms for these differences have been proposed, including varying diffusion rates (secondary to increased liver volume in females), altered body fat composition, lowered conjugation capacity in females and/or alternative mechanisms of uptake (Chang *et al.* 2000; Coldham and Sauer 2000; Schlosser *et al.* 2006). These species- and gender-based differences may contribute to differences in biological activity and indicate caution is required when extrapolating between studies.

	Rats		Dogs	
	Unbound	Conjugated	Unbound	Conjugated
Dose (mg/kg BW)	nmol/kg tissue			
50	734 - 1449	966 - 3235	280 - 284	1287 - 1008
150	Not measured	Not measured	799 - 2794	2245 - 5865
500	7888 - 8625	9910 - 23492	1619 - 3873	4634 - 9728

Table 1.2. Liver tissue content of genistein following 1 month oral exposure (McClain *et al.* 2005; McClain *et al.* 2006b)

Age-related effects have also been observed in liver genistein concentration following 5 weeks of genistein exposure (Chen and Bakhiet 2006). Older rats appeared to retain less genistein than younger rats at higher doses (Chen and Bakhiet 2006), which may be related to hepatic fat content or metabolism. Greater hepatic isoflavone storage in younger animals may indicate their greater susceptibility to isoflavone-induced hepatic changes.

1.3.6.3. *Reproductive tract isoflavone content*

As the primary focus of isoflavone activity has been their influence in the reproductive tract, it is pertinent to determine the concentration of absorbed isoflavones that are deposited in the reproductive tissues. However, it must be noted that reproductive function is controlled by a complex series of interactions between the brain, endocrine system and reproductive tissues, so that influence may occur at any point along this axis, and low gonadal isoflavone concentrations do not rule out interference.

The concentration of genistein has been found to be significantly higher in reproductive organs than in peripheral organs (Coldham and Sauer 2000), but lower than the liver (Chang *et al.* 2000). Female reproductive organs typically have greater total isoflavone concentrations than males, but more importantly, the fraction of unbound forms sequestered in female organs was significantly greater than that of male organs (Chang *et al.* 2000; Coldham and Sauer 2000). The relatively high content of unbound genistein in female reproductive tissues may reflect a preferential partitioning of the fraction escaping first-pass metabolism in this tissue (Chang *et al.* 2000; Coldham and Sauer 2000), which is also observed in the liver. An alternative explanation is that local deconjugation may occur, similar to that seen for endogenous oestrogen sulphate conjugates (Cimino *et al.* 1999; Coldham and Sauer 2000; Chang *et al.* 2000). The higher lipid content of female reproductive tissues, compared to male tissues, may also be an important component in determining the observed gender-bias (Chang *et al.* 2000). Regardless of the mechanism,

the greater accumulation of isoflavones in female reproductive tracts may be associated with greater biological potential than in males.

Some studies have reported only low uterine and hepatic concentrations of unbound isoflavones and considered tissue exposure in these cases to be unlikely to confer physiological effects (Gu *et al.* 2005; McClain *et al.* 2005). However, other *in vitro* studies indicate that some activity may be apparent even at low tissue concentrations (Woclawek-Potocka *et al.* 2005ab). Further, higher tissue concentrations are reported by others to occur following *in utero* or lactational exposure, compared to dietary exposure (Chang *et al.* 2000), indicating the potential for enhanced activity in neonates.

1.4. Important Factors Affecting Biological Exposure and Effects of Isoflavones

Any factor able to exert influence on the absorption, metabolism, distribution, excretion or bioavailability of isoflavones has the potential to alter the resultant physiological response. Therefore, these factors must be considered when evaluating studies investigating the biological activity of isoflavones. Furthermore, these factors are important when comparing studies of isoflavone absorption, metabolism, bioavailability and excretion, in order to ensure reliable comparisons are made between isoflavones, populations and species.

1.4.1. Factors Affecting Absorption

A variety of dietary factors are known to influence nutrient absorption and the presence of food in the GIT is known to affect isoflavone absorption due to the stimulation of peristalsis, release of bile acids and/or presence of other interfering or binding substances (Piskula 2000). Therefore, studies administering isoflavones to fasted animals are likely to produce different pharmacokinetic data compared to studies in which fed animals were used. Components of the diet in which isoflavones are consumed, and the structure of the food itself are also likely to influence the availability of isoflavones for absorption (Setchell and Cassidy 1999; Andlauer *et al.* 2000a; Setchell *et al.* 2005; Eason *et al.* 2005; Steensma *et al.* 2006). For example, greater excretion rates have been reported for isoflavones within liquid matrices (Setchell 2000; Qiu *et al.* 2005; Cassidy *et al.* 2006; de Pascual-Teresa *et al.* 2006). The binding of isoflavones to dietary protein or fibre also appears to render them less available for absorption, or to stimulate divergent metabolism/excretion (Zhang *et al.* 1999; Andlauer *et al.* 2000a; Setchell *et al.* 2001; Uehara *et al.* 2001). Dietary fibre may further reduce isoflavone absorption via its effect on gut transit time, faecal bulk and microbial populations (Zhang *et al.* 1999; Bueno *et al.* 2000; Blakesmith *et al.* 2005). A longer residence time in an intestinal

environment containing degrading bacteria also reduces the amount of isoflavone available for absorption (Zheng *et al.* 2003 and 2004).

However, gut microflora and motility are both known to vary between individuals, and this is likely to influence the absorption and excretion of isoflavones, independent of diet (Lampe *et al.* 1998; Zhang *et al.* 1999; De Boever *et al.* 2000; Hur *et al.* 2000; Pino *et al.* 2000; Uehara *et al.* 2001; Rafii *et al.* 2003). Overall, the variation reported in pharmacokinetics between species may be a reflection of either a variable background diet or divergent GIT system. Therefore, caution is needed when extrapolating results between studies.

Andlauer *et al.* (2000a) suggest that food-related variation in isoflavone absorption may also be influenced by the different patterns of conjugation seen in foods (i.e. aglycone, glycosylated or malonyl-glycosylated). Yet, disparity exists as to the relative bioavailability of isoflavones when ingested in their conjugated (glycosidic), or aglycone forms. Some studies demonstrate ingested aglycones are absorbed faster and in higher amounts than glycosides (King *et al.* 1996; Sfakianos *et al.* 1997; Cimino *et al.* 1999; Andlauer *et al.* 2000b; Izumi *et al.* 2000; Setchell *et al.* 2001; Cassidy *et al.* 2006; Kano *et al.* 2006; Steensma *et al.* 2006), which is thought to reflect the need for hydrolysis of the glycoside moiety prior to absorption (King and Bursill 1998; Setchell *et al.* 2001; Turner *et al.* 2003). But other studies suggest that it is the glycosides that have a greater overall bioavailability, perhaps as a consequence of the glycoside moiety protecting the isoflavone skeleton from biodegradation prior to hydrolysis and absorption in the small intestine (Setchell *et al.* 2001; Ismail and Hayes 2005). Still, other studies report no difference if sufficient sampling points are included (King *et al.* 1996; Sfakianos *et al.* 1997; Zubik and Meydani 2003; Tsangalis *et al.* 2005). A temporal delay in absorption should not be confused with lower bioavailability, and prudence is needed when comparing bioavailability studies (e.g. Izumi *et al.* 2000; Richelle *et al.* 2002; Zubik and Meydani 2003; Kano *et al.* 2006).

Additionally, urinary, faecal and plasma concentrations of isoflavones exhibit dose-responsive patterns (Wiseman *et al.* 2004). The reduced bioavailability of isoflavones consumed in large quantities may be due to limited intralumenal substrate hydrolytic capacity, or a rate-limited uptake of the unbound forms (Setchell 2000; Setchell *et al.* 2002). However, the threshold for apparent saturation of various pharmacokinetic parameters with increasing dose is the subject of discussion (Chang *et al.* 2000; Bloedon *et al.* 2002; Coldham *et al.* 2002b; Cassidy 2005) and a multitude of other factors may be confounding.

Measurement of the urinary excretion of genistein as an estimate of its absorption is now known to be particularly misleading since the majority of ingested genistein may be retained in the EH circulation (Sfakianos *et al.* 1997). Therefore low concentrations of genistein accounted for in the urine do not necessarily infer poor absorption or bioavailability. Moreover, the proportion of the ingested dose occurring in the urine is typically estimated from pooled urine samples, while plasma is sampled at discrete time points. Hence, overestimation of total amount absorbed may occur if an isoflavone is measured twice, i.e. in the plasma at a time point within the pooling period and then again in the urine.

1.4.2. Factors Affecting Metabolism

As observed for their absorption, the presence of food in the GIT also appears to affect isoflavone metabolism, with studies showing more sulphates and less glucuronides were detected in the circulation of food-deprived rats (Piskula 2000). In addition, a greater inclusion of vegetables and/or carbohydrates in the diet is thought to increase the fermentation and biotransformation of isoflavones *in vivo*, possibly as a result of the hydrogen and short chain fatty acids produced during intestinal transit of these food types (Uehara *et al.* 2001; De Boever *et al.* 2000; Rafii *et al.* 2003; Clavel *et al.* 2005; Decroos *et al.* 2005; Setchell and Cole 2006). Increased consumption of meat has also been

associated with changes in isoflavone biotransformation (Hedlund *et al.* 2005), although interspecific differences are reported in this regards (Musey *et al.* 1995).

Age and gender have been demonstrated to influence the metabolism of isoflavones. Although inconsistent results are reported, it is thought that isoflavones may exhibit greater bioavailability in females than males (Coldham *et al.* 2002b), and that neonates may be unable to metabolise and/or excrete isoflavones to the same extent as adults (Setchell *et al.* 1998; Doerge *et al.* 2002; Atkinson *et al.* 2005; Cassidy 2005).

Isoflavone-specific and inter-individual differences in metabolism are reported. A greater proportion of daidzein and equol were found conjugated to both sulphate and glucuronic acid moieties than genistein while, conversely, genistein diglucuronides were more common than those of daidzein (Karakaya *et al.* 2004). The pattern of equol-producer status observed among human families is indicative of an autosomal dominant inheritance trait, although segregation analysis proved inconclusive for the metabolite *O*-DMA (Frankenfeld *et al.* 2004a). Non-genetic influence over equol production has also been reported. For example, the relative production of total equol from daidzein was inversely correlated with the degree of processing that the diet had undergone, and the plasma concentration of unbound forms increased linearly with increased processing (Slavin *et al.* 1988; Allred *et al.* 2005). The reduced presence of glycosides in highly processed soy foods may have influenced daidzein metabolism due to reduced competition for β-glycosidases and/or phase II metabolising enzymes (Allred *et al.* 2005). Other environmental factors which may affect equol-producing ability require further investigation as these are likely to influence the biological response of animals to daidzein.

Certain gastrointestinal bacteria are known to be stimulated by exposure to isoflavones (De Boever *et al.* 2000; Clavel *et al.* 2005) such that prolonged intake has been associated with altered isoflavone metabolism and/or absorption compared to single bolus doses (Lu

and Anderson 1998; Rafii *et al.* 2003; Hedlund *et al.* 2005). However, no induction of metabolic enzymes or difference in equol producer status was observed following previous isoflavone exposure in other studies (Setchell *et al.* 2003b; Frankenfeld *et al.* 2005; Mathey *et al.* 2006; Soucy *et al.* 2006; Védrine *et al.* 2006). The variable period of exposure and use of urinary *versus* plasma concentrations complicates interpretation and comparison of these studies.

Exposure to isoflavones via routes that do not include GIT transit is likely to elicit divergent metabolite profiles, compared to those seen in orally-exposed animals (Doerge *et al.* 2002). Nonetheless, some conjugation has still been apparent even after parenteral exposure. Serum concentrations of up to 35% unbound genistein have been observed following sub-cutaneous administration of genistein aglycone (Cotroneo *et al.* 2001; Doerge *et al.* 2002). The apparent conjugation of 65% of the serum content indicates significant conjugation at the tissue site, or may be the result of EH metabolites which have been secreted back into circulation rather than the bile. Interestingly, the ratio of total-to-free genistein does not vary over time following I/V exposure whilst oral exposure results in significant temporal changes (Cave *et al.* 2007a), potentially indicating a saturation threshold in intestinal metabolism.

Methodological factors are also important. In order to estimate the concentration of glucuronide metabolites, samples may be hydrolysed with a glucuronidase without sulphatase activity. Hydrolysis of only the glucuronic moieties in the absence of sulphatase (or *vice versa*) would only partially hydrolyse sulpho-glucuronide conjugates and the resulting mono-conjugates (with attached sulphate moieties in the former case) would not be extracted from the aqueous phase (Shelnutt *et al.* 2000). Therefore, the presence of mixed conjugates is likely to underestimate the total recovery (Shelnutt *et al.* 2000) and/or bioavailability. This in turn may lead to erroneous predictions of their biological activity.

1.4.3. Factors Affecting Excretion

Genistein's hydrophobic nature may promote its greater capacity for biliary recovery (Sfakianos *et al.* 1997). Likewise, daidzein may be less efficiently glucuronidated in some species, possibly due to the presence of 3 free hydroxyl groups on the genistein skeleton conferring it with greater opportunity for conjugation than daidzein (Karakaya *et al.* 2004; Allred *et al.* 2005). Interestingly, the enhanced sulphation of daidzein is postulated as a mechanism by which this isoflavone is more rapidly excreted in the urine than genistein (Sfakianos *et al.* 1997).

On the other hand, the 5-OH group, as well as hydroxyl groups at the 7 and 4' positions of genistein have been postulated to render it more susceptible to C-ring cleavage, compared to daidzein (Xu *et al.* 1995; King 1998; Simons *et al.* 2005; Das and Rosazza 2006). Hence, daidzein may be more likely to pass into the colon relatively unmodified, where metabolism to alternative forms (including equol and *O*-DMA) may occur without significant degradation.

The excretion of daidzein, equol and total isoflavonoid concentration is typically dose-responsive, although genistein and *O*-DMA do not appear to be particularly sensitive to dose (Slavin *et al.* 1998; Tsangalis *et al.* 2005). Interestingly, when assessed on a fractional basis, the percentage of ingested dose excreted in urine appears to decline with increasing dose, which is indicative of a saturable absorption or excretion mechanism (Setchell *et al.* 2003ab; Tsangalis *et al.* 2005).

In addition to isoflavone-specific differences, interspecific differences in the urinary excretion of isoflavones have also been reported. A significantly greater proportion of unbound forms were excreted by rats (52%), compared to humans (0.36%) (Cimino *et al.* 1999) and the proportion of sulphate conjugates in rat urine also appeared to be higher than that of humans (26% compared to 4%, respectively) (Cimino *et al.* 1999). However,

results were presented from only one human investigation and inter-assay variability was large (up to 61.3% CV) (Cimino *et al.* 1999) so further investigation is warranted.

It is important to note that EH recirculation is also likely to extend the biliary excretion of genistein beyond the initial intestinal transit of the dosing meal. Therefore, collecting faeces for a relatively short time, or including only the faeces known to be associated with an isoflavone-containing meal (i.e. through the use of an indicator dye ingested with the meal as in Xu *et al.* 1994), is likely to underestimate faecal excretion. Alternatively, dissimilar dietary factors and/or GIT characteristics may also result in different faecal isoflavone recoveries.

1.4.4. Factors Affecting Physiological Effects

As well as those factors influencing an animal's exposure to isoflavones, it is also important to consider the factors that have been shown to affect the strength, direction and nature of influence exerted by isoflavones *in vivo*. Comparison of studies in which one or more of these factors are different is relatively common, but should be interpreted with caution. Likewise, extrapolation of study findings should be limited to situations in which identical factors can be established. The effects of isoflavone activity in mammals are discussed in more detail a later section (see Section 1.5).

1.4.4.1. *Timing of exposure*

Evidence suggests that exposure during critical developmental periods produces different outcomes to those following post-pubertal administration (Medlock *et al.* 1995; Hollander 1997; Nagao *et al.* 2001; Souzeau *et al.* 2005). Puberty onset was modulated by oestrogens in an opposing manner, depending on the time of exposure, whereby prenatal exposure typically delayed puberty, but neonatal exposure resulted in precocious puberty (Levy *et al.* 1995). Neuroendocrine responsiveness is also thought to be

differentially programmed following exposure at different lifestages. Likewise, immature rodents may be more sensitive to oestrogenic compounds than OVX animals (Diel *et al.* 2002), while intact adult animals have shown lowered uterotrophic responses to isoflavone exposure compared to OVX animals (Cotroneo *et al.* 2001; Diel *et al.* 2006). This appears indicative of a further role for critical developmental periods in predicting isoflavone impact.

Isoflavone accumulation in the body appears to be significant during prenatal periods (Brown and Setchell 2001). Serum isoflavone concentrations in rat pups, born to dietary isoflavone-exposed dams, decreased significantly between birth and 6 days of age, but remained constant thereafter during lactational exposure (Brown and Setchell 2001). This suggests that *in utero* exposure may be a more important transfer pathway than lactational transfer.

Furthermore, it has been suggested that maternal metabolism may alter the metabolite profile and absolute level of isoflavone exposure of foetuses compared to neonates. Indeed, the metabolite profile of gestationally-exposed animals differed from that observed after lactational exposure (Brown and Setchell 2001; Todaka *et al.* 2005). In particular, circulating equol concentrations were significantly higher in newborn (i.e. gestationally exposed) *versus* suckling pups (Brown and Setchell 2001), and sulphate conjugation was higher in infants than their mothers (Todaka *et al.* 2005). The possibility that biotransformation of isoflavones in neonates is reduced, compared to adults (see Section 1.4.2.), may further contribute to the greater susceptibility to isoflavone induced changes during this lifestage.

1.4.4.2. In vivo *dose and* in vitro *concentration*

Genistein has remarkably biphasic effects, with opposing biological effects often observed according to the dose-range (Linassier *et al.* 1990; Hsu *et al.* 1999; Dang *et al.*

2003; Cappelletti *et al.* 2006; Hwang *et al.* 2006). The effects observed after administration of doses higher than typically achieved through a diet (pharmacological) have typically been different from those following lower (physiological) doses (Levy *et al.* 1995; Hilakivi-Clarke *et al.* 1998). Generally, genistein is known to act in an oestrogen-like manner when administered at low concentrations, and in an anti-oestrogenic manner at high concentrations, in both *in vivo* and *in vitro* studies (Hilakivi-Clarke *et al.* 1998; Dang *et al.* 2003; Cappelletti *et al.* 2006; Hwang *et al.* 2006). Furthermore, supra-physiological concentrations (500 μM) trigger primarily stress response genes *in vitro*, and show very little overlap with the genes triggered at lower concentrations (Konstantakopoulos *et al.* 2006). Whilst large acute doses may be effective in demonstrating oestrogenic potential, the often marked but transient effects observed in such studies (Brown and Lamartiniere *et al.* 2000), do not predict the effects of long-term exposure at lower doses.

In addition to the need to determine isoflavone efficacy at physiological concentrations *in vivo*, it is also necessary to define the metabolites under investigation. Many studies have used circulating *in vivo* serum concentrations as the basis for their *in vitro* dose range, but failed to account for the fact that reported serum concentrations are often the result of enzymatic deconjugation, and that the concentration of unbound isoflavone in circulation is substantially lower than that of the "total" reported concentration. Therefore, administration of purified aglycones in doses representative of total serum unbound equivalents is not physiologically-relevant. Furthermore, the circulating isoflavone profile is not simply limited to conjugated or deconjugated forms of the unbound compound. Some metabolites (e.g. equol and *O*-DMA) would not be detected using simple enzymatic assays for deconjugated total equivalents of genistein and daidzein, due to their alternative molecular structure. Yet these metabolites possess significant oestrogenic or anti-oestrogenic potential and their biological impact would not be considered in studies utilising only purified genistein or daidzein *in vitro*. Finally, as previously discussed, tissue concentrations are unlikely to be accurately reflected by plasma concentrations (see Section 1.3.6).

1.4.4.3. *Background ER and endogenous oestrogen environment*

The background oestrogenic environment is known to affect cellular responses to isoflavones, such that the functional effects of genistein differ between intact, cycling and OVX animals (with or without exogenous oestrogens) (Diel *et al.* 2006). Generally genistein has been found to be ineffective in producing uterotrophic or cellular growth responses in intact animals, yet was antagonistic (Mäkelä *et al.* 1995; Tansey *et al.* 1998; Erlandsson *et al.* 2005; Diel *et al.* 2006), or additive (Foth and Cline 1998; Wang *et al.* 2005) in the presence of exogenous or endogenous E_2.

Receptor expression modulation by isoflavones is also similarly dependent on the presence of E_2. Depending on the presence or absence of E_2, mixed antagonistic and agonistic effects were observed in the expression of various receptors in rodents following genistein exposure (Diel *et al.* 2006). Interestingly, the direction of P_4 receptor (PR) response was consistently agonistic and was independent of circulating oestrogen concentration (Diel *et al.* 2006), indicating a receptor-specific difference. However, the extent of response was greater when genistein was administered in combination with E_2, and indicated an additive, agonist effect of genistein on the PR (Diel *et al.* 2006). *In vitro* studies have determined that competitive binding of isoflavones to the oestrogen receptor (ER) is also modulated by the presence of E_2 (Hwang *et al.* 2006) and may explain the observed differences in response between intact and OVX animals.

The difference between ERα and ERβ-mediated effects may also induce differences in outcomes. An isoflavone's greater affinity for ERβ over ERα is likely to confer distinct and contrasting physiological responses according to the ER expression of the tissue affected (An *et al.* 2001; Bennetau-Pelissero *et al.* 2001; Ford *et al.* 2006; Chrzan and Bradford 2007). This may partially explain the pleiotropic nature of isoflavone activity, since ER distribution and concentration varies on a tissue-specific level (Barnes *et al.* 2000).

Additionally, isoflavones have demonstrated binding affinity for sex hormone binding globulin (SHBG), although this is significantly lower than that of endogenous E_2 (Brown and Setchell 2001; Hillerns *et al.* 2005). Therefore, the presence of high circulating concentrations of E_2 may result in the greater occupation of SHBG with E_2, leaving a greater concentration of isoflavones unbound to SHBG. Isoflavones may consequentially be capable of enhanced sex steroid receptor occupancy in this situation, compared to low E_2 environments (Nagel *et al.* 1998; Brown and Setchell 2001).

Finally, although OVX animals can be artificially oestrogenised through the administration of exogenous oestrogen, the absence of P_4 in OVX animals may be another reason for the different responses observed between intact and artificially oestrogenised OVX animals (Diel *et al.* 2006). This supports the idea that OVX animals do not make appropriate models of isoflavone activity under normal hormonal conditions *in vivo*. The interrelated effects of phytoestrogens and E_2 combinations are important considerations when extrapolating to "real-life" situations, where animals are exposed to a number of different dietary and environmental oestrogens (van Meeuwen *et al.* 2007). Direct comparison of oestrogenicity between studies should only include those conducted in age-matched animals of equivalent reproductive status, and should consider the relevance to a normal population.

1.4.4.4. *Cell and tissue specificity*

Substrate differences are important determinants of isoflavone activity, and may reflect the different binding modes within the ligand active sites. The cell lines and substrates used must be considered when comparing reported results of isoflavone exposure (Calderelli *et al.* 2005; Hertrampf *et al.* 2005; Brožič *et al.* 2006; Limer *et al.* 2006). However, isoflavone-specific differences may also play a role, with alternative pathways of action suggested between tissues and cell types (Limer *et al.* 2006).

The increased or differing activity (Brown *et al.* 1998), or the sensitivity to lower isoflavone doses (Lamartiniere *et al.* 1998; Delclos *et al.* 2001) observed for mammary tissue compared to other reproductive tissue is an example of this tissue-specificity. As shown in the liver (Limer *et al.* 2006) different expression of ERα *versus* ERβ in mammary tissue (Delclos *et al.* 2001) may be involved in modulating isoflavone activity. Non-classic target tissues may also be affected in the absence of uterine effects, with effects observed in one physiological end-point, but no modulation detected in others (Diel *et al.* 2000; Nakai *et al.* 2005).

1.4.4.5. *Species specificity*

Extrapolation of results from rodent studies to humans is common. However, published estimates of the half-life of genistein have varied between species, whereby rodents have been shown to have a greater excretory capacity for isoflavones than humans (see Section 1.3.4). Higher doses must also be used for rodents in order to produce serum concentrations equivalent to those seen in humans consuming significantly lower doses of isoflavones (Delclos *et al.* 2001; Jefferson *et al.* 2005 and 2007; van Meeuwen *et al.* 2007). Interspecific differences are further complicated by the possibility that results *in vitro* may not necessarily reflect those *in vivo* (Latonnelle *et al.* 2002). Furthermore, hepatic effects of isoflavones may be opposing and contrasting in different rat strains (Nakai *et al.* 2005), which may be due to different mechanisms of action, or altered response of the hepatic markers involved (Geis *et al.* 2005). Therefore, consideration of the potential for interspecific differences must be made when comparing studies utilising different animal models.

1.4.4.6. *Form of isoflavone*

Isoflavones have varying oestrogenic potencies and mechanisms of actions. The effect of one particular isoflavone cannot be extrapolated to another. Similarly, the form in which isoflavones are administered has direct implications for their biological potential.

The majority of soy's oestrogenic activities are related to its isoflavone content, and effects have not been observed in some studies when the isoflavone component of soy was removed (Drane *et al.* 1980). However, this is not always the case and the nature of effects, as well as their presence or absence, varies according to whether whole soy food or purified isoflavones were consumed (Anthony *et al.* 1996; Eason *et al.* 2005; McClain *et al.* 2006b). Interaction between genistein and other phytoestrogens in soy, as well as altered pharmacokinetics following purification, are likely to be responsible for these observed differences in biological activity.

Of the two main isoflavone types (genistein and daidzein), genistein is typically reported to be the more oestrogenic when tested against daidzein under various test conditions and for different measures of oestrogenicity (Wong and Flux 1962; Farmakaldis *et al.* 1985; Nikov *et al.* 1997; Kouki *et al.* 2003; Miller *et al.* 2003). Daidzein is known to undergo alternative metabolic and excretion pathways to genistein (see Section 1.3.3), and as such it is unlikely that equal amounts of hydrolyzed daidzein aglycone is absorbed and/or maintained in circulation, compared to the respective genistein aglycone. This will significantly reduce daidzein's bio-efficacy. However, in contrast, daidzein has also been shown to elicit growth stimulatory effects where genistein was incapable of doing so (Limer *et al.* 2006), or had weaker effects (Calderelli *et al.* 2005). Furthermore, estimation of daidzein potential activity must not be limited to standard uterotrophic effects, or its capacity for ER binding (Diel *et al.* 2000). Depending on the test system and the parameter measured, daidzein may act as either a potent or weak oestrogen relative to genistein.

Metabolic modification may influence isoflavone binding to either ER subtype or to other target proteins, and may be important in explaining the diverse array of responses elicited by isoflavones under different study conditions (Barnes *et al.* 2000). For example, maximum affinity for ER subtypes is typically displayed when 2 hydroxy groups are present on appropriate positions of the molecule. Reduction of daidzein to equol (by removal of the ketone oxygen at the 4-position, and double bond between C-2 and C-3 in the B-ring), results in a 7-fold increase in its affinity for both ER subtypes (Sathyamoorthy and Wang 1997; Branham *et al.* 2002; Lehmann *et al.* 2005; Hwang *et al.* 2006). Hydroxylation at the 3'-position does not affect binding affinity, whilst the 6-hydroxy metabolite has a significantly reduced binding ability (Lehmann *et al.* 2005).

Of similar importance is the type of equol utilised in studies. Equol exists as the enantiomers *R*-equol and *S*-equol, of which only the latter is produced from metabolic reduction (Muthyala *et al.* 2004; Wood *et al.* 2006a). However, the majority of synthetically-derived equol is a racemic mixture of both enantiomers. Muthyala *et al.* (2004) reported that *S*-equol demonstrated a clear (13-fold) preference for ERβ, whilst the *R*-equol enantiomer bound more weakly, and with a greater preference for ERα. However, Selvaraj *et al.* (2004) reported that both enantiomers had equal ERα and ERβ binding. Other studies using transcription assays have shown that enantiomer binding affinity was not correlated with potency, indicating differential interaction with cellular co-regulators and/or altered consequences of cellular metabolism (Muthyala *et al.* 2004). Laboratory studies using the *R*-enantiomer may therefore demonstrate oestrogenic activity that is not exhibited by endogenously derived equol.

Conjugation of isoflavones to sulphate, glucuronide or mixed glucuronyl and sulphonyl moieties had varied effects on their ER binding affinity and oestrogenic potencies (Kinjo *et al.* 2004). In general, sulphates were poor stimulators of cell growth, bound relatively poorly to human ER, and failed to induce significant oestrogen-dependent enzyme activity (Kinjo *et al.* 2004). Cellular stimulation was lowered when isoflavones were conjugated to glucuronic acid, but was equivalent to aglycone forms when both

glucuronyl and sulfonyl conjugation occurred (Kinjo *et al.* 2004). Interestingly, glucuronic acid conjugation increased binding affinity to ERα, when compared to aglycone forms of both genistein and daidzein, but glucuronidation of dihydrodaidzein reduced binding affinity considerably (Kinjo *et al.* 2004). Sulphation of genistein resulted in poor binding to both ERα and ERβ, although the 4'-0-sulphate bound slightly to ERβ (Kinjo *et al.* 2004).

Conversion of daidzein to *O*-DMA aglycone increased its binding affinity to ERβ substantially, so that it was observed to bind at levels equivalent to E_2 (Kinjo *et al.* 2004). This was reflected in the potent induction of ERβ-dependent enzymes, and strong stimulatory effect on growth by *O*-DMA *in vitro* (Kinjo *et al.* 2004). Glucuronidation of *O*-DMA reduced its affinity, and rendered binding levels comparable to the daidzein glucuronide and mixed daidzein glucuronyl-sulphate conjugates (Kinjo *et al.* 2004).

1.4.4.7. *Route of administration*

Differences in the efficacy of isoflavones administered via injection *versus* oral exposure are well reported in the literature (Cotroneo *et al.* 2001; Selvaraj *et al.* 2004; Eason *et al.* 2005; Lehmann *et al.* 2005). The varied biological potency of the metabolites produced during gastrointestinal transit, enteric and hepatic first-pass metabolism highlights the biological irrelevance of studies utilising any route other than oral administration of isoflavones.

Not only is the absolute exposure to isoflavone reduced after oral exposure, but the resultant circulating metabolite profile has been shown to contain a much lower proportion of aglycones than following parenteral exposure. For example, 35% unbound genistein was found in circulation after sub-cutaneous injection, but typically only 1-5% was detected after oral ingestion (Cotroneo *et al.* 2001; Selvaraj *et al.* 2004). Yet, whilst lower aglycone concentration may indicate lowered biological efficacy, some metabolites

produced after enteric or intestinal biotransformation possess a greater bio-activity potential than the parent compounds (as is the case for daidzein and equol). Furthermore, pooling of the administered compound at the site of injection is also possible, and may confer a slower release and potentially longer exposure period than following oral administration (Cotroneo *et al.* 2001). These effects render the use of parenterally administered isoflavones unreliable in predicting *in vivo* effects, following normal intake.

Likewise, similar serum genistein concentrations are seen in humans consuming soy meals and rats following sub-cutaneous administration of much higher doses (Jefferson *et al.* 2005). It has been argued that the administration of supra-physiological doses via sub-cutaneous injection is appropriate in this model as it produces circulating levels equivalent to that seen in humans after dietary exposure, and that the effect is the same regardless of the route of administration (Jefferson *et al.* 2005; van Meeuwen *et al.* 2007). Since intestinal deconjugation of orally ingested glycosides results in unbound isoflavone exposure after oral dosing, the idea that it is acceptable to administer the aglycone form sub-cutaneously (Jefferson *et al.* 2005; Jefferson *et al.* 2007) relies on the assumption that isoflavones undergo no further metabolic conversion after intestinal absorption. This suggestion is not supported by the literature (see Section 1.3.3).

In a study aimed at comparing sub-cutaneous *versus* oral administration of genistein, Jefferson *et al.* (2007) reported approximately 80% of the oral dose was absorbed into circulation, and resulted in a similar response to the sub-cutaneous dose that was 100% absorbed. From this they concluded that there was no difference between oral or sub-cutaneous dose routes. However, their results (Jefferson *et al.* 2007) did not support their conclusion. Furthermore, this study monitored only uterine weight, which is a relatively insensitive endpoint, and failed to examine effects in post-pubertal animals. It is pertinent to note that the majority of published work from this research group has focused on the post-pubertal induction of ER expression and incidence of abnormal ovarian differentiation. Hence, the findings of their comparative uterotrophic study should not have been extrapolated to their studies investigating alternative biological endpoints.

Finally, since serum profiles were reported as "total equivalents" which do not take into account the metabolite profile, it was also erroneous to compare circulating serum concentrations in this regard as metabolite profiles are likely to differ considerably and infer distinct biological activities.

Another important consideration is the observation that while sub-cutaneous injection provides a discrete daily bolus dose, *ad libitum* dietary exposure provides the dose over a 24 h period, in much smaller amounts, but at a more constant or frequent rate. This altered temporal delivery is likely to have significant influence over the pharmacokinetics and potential biological activity of compounds, but is seldom mentioned as a limitation to sub-cutaneous administration (the exception being Selvaraj *et al.* 2004). A similar criticism is necessary for the provision of a single dietary bolus, although it is perhaps more justifiable given that humans and many captive carnivores (e.g. cheetahs, dogs and cats) are likely to consume discrete meals throughout the day.

Significant anabolic effects were observed in rats just 6 h following intraperitoneal injections of genistein (calculated to be approximately 13.3 mg/kg BW) (Noteboom and Gorski 1963). This effect could not be replicated following oral consumption of similar or higher doses (McClain *et al.* 2006b). If the maximum concentration (C_{max}) of isoflavone exposure is more important than steady-state plasma concentration in providing isoflavone oestrogenic activity, this would provide another explanation for enhanced activity following sub-cutaneous injection compared to oral intake. To this end, the rapid onset of oestrogen-induced changes after parenteral administration of genistein (Noteboom and Gorski 1963), lends support to the importance of C_{max}, as opposed to overall fraction of dose absorbed. In this regard the investigation of C_{max} following discrete dietary exposure provides a relevant indicator of biological potential in species consuming distinct meals.

1.4.4.8. *Duration of exposure and monitoring*

Since the duration of exposure could play a major role in the nature and extent of observed isoflavone-induced changes, it is surprising how few long-term studies have been published. This is especially remarkable considering the reliance on animal models to support claims for soy-induced health benefits, which were inferred by epidemiological studies comparing life-time soy consumption in Asian countries to the low soy diets consumed in Western countries.

Furthermore, effects may not become apparent for some time following exposure (Medlock *et al.* 1995; Hollander 1997). Hence, monitoring must be extended beyond sexual maturity in order to ensure effects of neonatal exposure are not missed. No abnormal health indicators were observed after a single, acute oral exposure of 2000 mg genistein/kg BW in rats, and the oral lethal dose was reported to be >2000 mg genistein/kg BW (McClain *et al.* 2006b). However, longer exposure resulted in the appearance of detectable changes, even at lower doses (McClain *et al.* 2006b; Whitten *et al.* 1995). In a multiple-dose, sub-chronic exposure study, the incidence and severity of observed changes in genistein-treated groups increased in a time-dependent manner (McClain *et al.* 2006b).

Alternatively, some effects were only apparent immediately after exposure, and monitoring at later time points or after continued exposure resulted in no observable changes (Mäkelä *et al.* 1995; Awoniyi *et al.* 1998; Jefferson *et al.* 2002a; Nikaido *et al.* 2004; McClain *et al.* 2006b). Similar transient effects following acute exposure have also been noted in the modulation of growth factors, uterine hypertrophy (Brown and Lamartinere 2000), expression of ERα protein (Cotroneo *et al.* 2001), and reduction of corpora lutea (Nikaido *et al.* 2004). This is suggestive of acute responses at non-critical development stages and/or powerful homeostatic mechanisms capable of abrogating short-term perturbations. The transient nature of some isoflavone effects has

ramifications when attempting to elucidate the biological consequence of acute *versus* chronic exposure.

1.4.4.9. *Background diet and prior exposure*

When evaluating the oestrogenic potential of isoflavones in whole diets it is important to consider confounding factors, such as other phytoestrogens, or compounds with oestrogenic potential, as well as the nutritional composition of the diet. For example, certain mycotoxins (e.g. zearalenone) possess oestrogenic properties. However, only one study screened diets for this contaminant before investigating the potential effects of soy (Drane *et al.* 1980). Furthermore, soy ingredients are not the only source of phytoestrogens in feedstuffs and significant concentrations of the highly oestrogenic compound, coumestrol, are likely to occur in alfalfa-containing diets (USDA-Iowa Database 1999). Coumestrol can stimulate induction of the P_4 receptor in the uterus, accelerate the onset of puberty, and cause oestrous cycle irregularities after acute and chronic administration (Whitten and Naftolin 1992; Whitten *et al.* 1993). Similarly, studies of soy diets compared to purified isoflavones that failed to provide an isoflavone-free soy diet group cannot reliably demonstrate any specific causative effect of isoflavones.

The oestrogenic potential of a large number of commercially-available laboratory animal diets introduces the potential for significant interference in results if they are utilised as control diets against which compounds are assessed (Brown and Setchell 2001). Screening control animals for isoflavone exposure is not common, although control groups are occasionally reported to have detectable isoflavones concentrations in plasma, serum and/or urine (Brown and Setchell 2001; White *et al.* 2004; McClain *et al.* 2006b).

Even in instances where studies have ensured that the control or basal diet is free of the particular compound(s) under investigation, control groups in some studies have still

exhibited circulating isoflavones (e.g. White *et al.* 2004; McClain *et al.* 2006b). It is possible that exposure to a soy- or alfalfa-containing diet prior to the treatment phase (often unknown to the authors themselves), resulted in residual circulating isoflavones. These isoflavones may originate from tissue stores, or may simply be a result of the extended half-life exhibited by some isoflavones (see Section 1.3.2). Placental, lactational or neonatal exposure to isoflavones may also modulate responses to oestrogen insult later in life. Therefore, using a control group with prior or concurrent exposure to isoflavones may underestimate treatment effects, or alter control group responses.

Another concern is the unidentified sources of isoflavones present in the diet, as detected by White *et al.* (2004) (see Section 1.2.1.2). It is possible that the low isoflavone content of the control diet, combined with the lack of prior dietary knowledge of the cats utilised in this study (some entered the study with positive urinary isoflavone contents), could explain the modest differences reported between control and test groups in the parameters tested. Inclusion of cats with varying prior exposure to isoflavones, and possibly altered metabolic capacity, may well have dampened the between-group differences.

Investigation of only a limited number of isoflavones also fails to consider the impact that other undetermined phytoestrogens may have on circulating isoflavone concentrations. For example, screening for only genistein may leave unidentified concentrations of daidzein capable of significant biological activity. Likewise, inhibitory effects of dietary lignans on genistein activity in bone have been reported (Power *et al.* 2006). Precursors to genistein and daidzein (biochanin A and formononetin, respectively) may not be screened for in trial diets, yet substantial biotransformation of these substances could occur *in vivo*, effectively increasing genistein and daidzein exposure to a concentration that would not be anticipated from dietary analyses.

Despite the evidence for potential and observed interference, the impact of low-phytoestrogen exposure through the use of standard laboratory rodent chow as a control

diet is still controversial. A number of studies have shown that significant differences between control and treatment animals were still detectable despite having utilised an isoflavone-containing control diet (Newbold *et al.* 2001; Jefferson *et al.* 2005 and 2006). The finding of a 35% incidence of uterine adenocarcinoma in animals exogenously-exposed to genistein, compared to a lack of incidence in the control group consuming a soy- and alfalfa-containing commercial diet of low phytoestrogen content (Newbold *et al.* 2001) suggests this isoflavone-containing control diet exerted little, if any, oestrogenic effect. However, the use of supra-physiological doses administered sub-cutaneously in the study by Newbold *et al.* (2001) was likely to have overridden any subtle dietary effect. In another study, monitoring was not continued post-pubertally and significant perturbations may have been missed (Naciff *et al.* 2004). Furthermore, histology was assessed in only a small number of animals following short-term exposure during non-critical periods of development (Naciff *et al.* 2004).

Arguments against the use of phytoestrogen-containing control diets cite evidence of the uterotrophic effects of standard chow diets (containing >400 ppm isoflavone, which provides animals with approximately 1.6 mg isoflavones per day) (Wang *et al.* 2005). Vulvar carcinomas have also been observed in animals fed commercial diets containing naturally-occurring phytoestrogens, as well as those containing modified soy content (Thigpen *et al.* 2001).

In addition, hormonal steroids may also enter the diet via circulating concentrations present in meat. Daxenberger *et al.* (2001) describe the concentration of endogenous E_2, and its metabolites in cattle and poultry meat as being in the range of 1.3 – 3690 pg/g, with potential biological activity when consumed at the high end of this range. It is therefore possible that non-isoflavone steroids in the background diet of meat-eating animals and human subjects may have interfered with the results of some intervention trials. However, modification/denaturing of mammalian steroids by processing/cooking was not reported (Daxenberger *et al.* 2001), and Setchell *et al.* (1987ab) did not determine any influence of the horsemeat devoid of plant steroids in their rodent

bioassay. This source of potential oestrogenic compounds is therefore assumed to be minimal.

1.4.4.10. *Energy intake and weight gain*

Neonatal and pre-pubertal weight gain and metabolisable energy (ME) intake affect puberty onset, and exert uterotrophic effects in the absence of isoflavones (Odum *et al.* 2004; Dunger *et al.* 2006). Rapid early weight gain resulted in higher insulin-like growth factor 1 (IGF-1) concentrations in children, possibly due to early induction of growth hormone receptors (Dunger *et al.* 2006). Similarly, uterotrophic effects induced by increasing ME intake were abolished by co-administration of a gonoadotrophin releasing hormone (GnRH) antagonist, but the effects of diethylstilbestrol were unaffected (Odum *et al.* 2004). This is suggestive of different physiological interference pathways (Odum *et al.* 2004). It is hence, possible that disparity in reproductive developmental effects observed between experimental studies may be have been due to effects on weight gain, rather than effects of oestrogenic dietary components.

1.4.4.11. *Genotype, phenotype and gender-based differences*

The molecular effects and causes of different isoflavone activities is an emerging area of science. Polymorphisms of certain genes may explain a degree of the variation observed in the responses elicited by isoflavones (discussed later in Section 1.5). Polymorphisms of ERs have been associated with greater isoflavone-induced effects (Hall *et al.* 2006; Hedelin *et al.* 2006; Tsuchiya *et al.* 2007), and equol-producer status is a mechanism by which daidzein may exert its variable response in different phenotypes (Nettleton *et al.* 2005b; Low *et al.* 2005).

Equol's greater affinity for both ERs compared to daidzein requires that equol-producer status be identified, and equally distributed, amongst study populations. However, this is

not always reported in studies. Oestrogen metabolism, regardless of isoflavone intake, differs according to certain human genotypes, and (for example) depends on the presence of specific polymorphisms of CYP1A1 and CYP1B1 alleles (Sowers *et al.* 2006). Other genotypes and/or phenotypes may also be involved but have yet to be identified. These underlying differences may explain, in part, the variable results in steroid metabolism following isoflavone exposure.

The tissue and parameter measured also appears to be important since some parameters are unaffected by equol-producer status (Frankenfeld *et al.* 2004b; Nettleton *et al.* 2005a; Hall *et al.* 2006). Additionally, the timing of soy challenges administered to determine equol-producer phenotype should be performed concurrently with the collection of comparative data, unlike the study of Frankenfeld *et al.* (2004b) in which 2 – 3 years elapsed between phases and dietary changes may have modulated isoflavone metabolic capacity.

Gender-based differences are well reported in the literature. Influence of phytoestrogens on sexual behaviour (Whitten *et al.* 1995), serum hormones, reproductive/fertility parameters (Ryökkynen *et al.* 2005 and 2006), and alterations in 5α-reductase activity (Laurenzana *et al.* 2002) have all been reported to be more pronounced in the male than female. Isoflavone-related effects on 2α-OH-testosterone, 16α-OH-testosterone, glucose concentration, and/or CYP450 expression have also been demonstrated to be more marked in males than females (Laurenzana *et al.* 2002; McClain *et al.* 2006b). However, other studies have reported opposite effects, whereby steroid hormone profile modulation was more pronounced in the female than the male (Badger *et al.* 2001; Malaivijitnond *et al.* 2004).

The high circulating concentration of endogenous E_2 in females may mask any effects of genistein, unlike the situation in males, where genistein has been shown to exert demonstrable effects in some tissues (McClain *et al.* 2006b). The variable metabolism

and tissue distribution of isoflavones in males and females may also offer some explanation for these findings. However, in other respects females appeared to be more susceptible to genistein activity, and exhibited modulation of serum hepatic enzyme concentrations where no changes were detected in males (McClain *et al.* 2006b). Similar gender-based differences have been shown for daidzein which did not modulate male expression of ERα, but was able to do so in female hepatocytes (Bennetau-Pelissero *et al.* 1998). Denser ER populations in female tissue may render them more sensitive to isoflavone activity (Bennetau-Pelissero *et al.* 1998).

The direction of isoflavone effects in the liver have been reported to be influenced by the presence or absence of circulating E_2 (Diel *et al.* 2006). In intact animals, genistein was a strong modulator of oestrogen-dependent gene expression, with more pronounced effects seen compared to gonadectomised animals (Diel *et al.* 2006). This was possibly due to OVX-induced oestrogen depletion of the liver (Diel *et al.* 2006). Oestrogen receptor-α expression was increased in females fed a diet that contained 1250 ppm genistein, but decreased in males (Laurenzana *et al.* 2002). In the study by Laurenzana *et al.* (2002) genistein failed to modulate female ERα expression. However, in this model, oestrogen produced the opposite effect in male ERα expression. Gender-related effects are therefore important considerations when extrapolating findings between studies.

1.5. Effects of Isoflavones in the Female Reproductive Tract of Non-Felid Mammals

1.5.1. Background

Although the use of herbal remedies to assist fertility and contraception dates back to the days of Hippocrates (460 – 377 B.C.) (Dubick 1986) it took a further 1500 years before the oestrogenic effects of phytoestrogens were scientifically recognized. Due primarily to their structural similarity to endogenous oestrogens (see Figure 1.5), isoflavones have become popular targets for scientific investigation of their potential beneficial and detrimental physiological effects, in a wide variety of species.

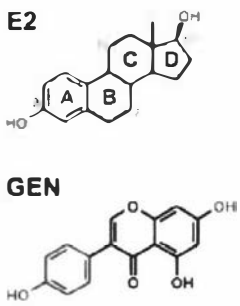


Figure 1.5. Structure of oestradiol (E2), and genistein (GEN). Green rings are the phenolic A-rings; ring classification (A – D) are depicted in E_2 (from Pike 2006).

The oestrogenic activities of isoflavones allow them to inhibit reproductive organ growth and to interfere with reproductive performance in a number of species. Reports of reproductive effects following isoflavone exposure include the induction of oestrus, promotion of gonad growth in female consumers, binding to oestrogen receptors, induction of oestrogen-responsive gene products, stimulation of oestrogen receptor (ER)-positive cell growth, and binding to carrier proteins, thereby reducing their availability to transport endogenous hormones (Santell *et al.* 1997; Kurzer and Xu 1997; Andlauer *et al.* 2000b; Strom *et al.* 2001). Additionally, isoflavones may react directly with endogenous hormones, alter hormone breakdown or synthesis, inhibit enzyme activity and/or

modulate gene expression (Hollander 1997). However, isoflavones are not always observed to act as classical oestrogens, or to have consistent effects between tissues (Barnes *et al.* 2000). Hence, the ultimate effect of isoflavones varies with form, dosage, route of administration, tissue type, species, gender and response criteria under investigation (Setchell and Cassidy 1999; Davis 2001). These differences may relate to disparities in factors affecting bioavailability, as discussed previously, or may be more directly related to the plethora of isoflavone activities in the reproductive system.

1.5.2. Reproductive Consequences of Isoflavone Administration

The biological consequences of isoflavone administration remain controversial. Many conflicting reports exist as to their safety and efficacy as health-promoting agents and/or as inducers of detrimental physiological endpoints. Some studies indicate that, when provided at doses achievable from dietary sources, isoflavones are not considered to be harmful to reproductive parameters in adult animals (McClain *et al.* 2005 and 2006ab). However, exposure at critical developmental stages, or in concentrations at the high end of the dietary range, has produced different responses to those seen in animals exposed at different life stages or to lower doses. To this end, permanent and irreversible changes in reproductive physiology were observed in sheep consuming phytoestrogens via subterranean clover ("clover disease") (Bennetts *et al.* 1946). Indeed, it was the findings of infertility in these animals that first sparked concerns as to the oestrogenic potential of dietary isoflavones (Bennetts *et al.* 1946).

1.5.2.1. *Uterotrophic action*

Assessment of oestrogenic activity typically includes uterotrophic assays, as these are considered the gold standard for assessment of *in vivo* oestrogenic activity (Clode 2006). An increase in uterine wet weight has been observed after isoflavone consumption in a variety of species (Drane *et al.* 1980; Setchell *et al.* 1987ab; McClain *et al.* 2005; Ford *et al.* 2006; McClain *et al.* 2006b; van Meeuwen *et al.* 2007) and uterotrophic effects are

known to occur following gestational, neonatal, pre-pubertal and post-pubertal exposure, with varying severity. However, the permanence, severity, incidence and nature of uterotrophic changes are typically dose- and time-related.

Additionally, findings are not consistent within the literature, and uterine wet weight has not always increased following isoflavone exposure. In fact, decreases in uterine and ovarian weight have also been reported (Awoniyi *et al.* 1998; Lamartiniere *et al.* 1998; Kouki *et al.* 2003; Erlandsson *et al.* 2005). A number of other investigations have failed to demonstrate any change in uterine weight or histological parameters of the reproductive tract and mammary gland (Foth and Cline 1998; Tansey *et al.* 1998; Naciff *et al.* 2004; Nikander *et al.* 2005; Eason *et al.* 2005; Nikaido *et al.* 2005; Rauš *et al.* 2006; Wood *et al.* 2006ab).

Other physiological changes have occasionally been observed in the absence of uterotrophic effects, such as acceleration in puberty onset (Badger *et al.* 2001; Nikaido *et al.* 2005), or favourable modifications of plasma lipid and lipoprotein concentrations (Anthony *et al.* 1996). Therefore, it appears that uterotrophic evaluation including only comparative wet weight measurements may not always be appropriate for the detection of oestrogenic activity. Indeed histological, cytological and molecular changes are known to occur after exposure to some isoflavones in the absence of uterotrophic effects (Diel *et al.* 2002; Jefferson *et al.* 2002b; Selvaraj *et al.* 2004; Hertrampf *et al.* 2005). As such, the sensitivity of uterotrophic assays should be improved by including histological, molecular and biochemical endpoints (Diel *et al.* 2002; Clode 2006).

1.5.2.2. *Histological changes*

A diverse array of morphological, histological and molecular changes have been associated with isoflavone intake, including hypertrophy, hyperplasia, metaplasia, cyst formation, dilatation, cornification, atrophy and proliferation of the cells, tissues, organs

and glands of the reproductive tract (McDonald 1995; Gallo *et al.* 1999; Brown and Lamartiniere 2000; Delclos *et al.* 2001; Nagao *et al.* 2001; Newbold *et al.* 2001; Thigpen *et al.* 2001; Diel *et al.* 2002; Jefferson *et al.* 2002b; Unfer *et al.* 2004; Ford *et al.* 2006; McClain *et al.* 2006b). Modifications in the distribution, development and size of ovarian follicles have also been associated with soy and genistein exposure in rodents (Awoniyi *et al.* 1998; Gallo *et al.* 1999; Jefferson *et al.* 2002a; Masutomi *et al.* 2003). However, altered ovarian histology does not necessarily demonstrate impaired ovulation. For example, the number of corpora luteal (CL), which are indicative of ovulation, was unaffected by isoflavones in some studies (Lamartiniere *et al.* 1998; Gallo *et al.* 1999; Masutomi *et al.* 2003). In contrast, CL numbers were reduced after exposure to genistein in other studies (Awoniyi *et al.* 1998; Nagao *et al.* 2001; Kouki *et al.* 2003; Nikaido *et al.* 2004; Jefferson *et al.* 2005). Discrepancies may arise following divergent exposure regimes or may suggest unknown interactions and influential variables.

Follicles containing more than one oocyte (termed multioocyte follicles, MOFs) have been consistently observed in mice exposed to isoflavones (Jefferson *et al.* 2002a). The long term consequences of MOF production may include the premature depletion of oocytes, and a lowered fertility rate (Jefferson *et al.* 2002a). However, it is unclear whether the production of MOFs affects oocyte quality (Jefferson *et al.* 2006). Therefore, the presence of MOFs is of unknown consequence to short-term fertility.

Multioocyte follicles are thought to result from the failed separation of primary follicular cells from primordial oocytes during initial follicle organisation, whereby fewer oocytes undergo normal apoptosis (Nagao *et al.* 2001; Jefferson *et al.* 2002a and 2006; Takashima-Sasaki *et al.* 2006). Genistein's modulation of cell-signalling and programmed cell death are possible factors (see Section 1.5.2.6) in this distorted differentiation. However, the mechanism by which these isoflavone-induced effects are mediated is not fully understood, and the dose-dependent data emphasises the need to ensure extrapolation is limited to doses achievable via dietary intake.

Macroscopic changes in the reproductive tract have been reported in dogs following genistein exposure (McClain *et al.* 2005). However, these were ascribed to normal variation, since they reflected changes typically observed in dogs on a control diet at a testing facility (McClain *et al.* 2005). Yet, this control diet was reported to contain soybean meal, and although the daily genistein exposure of control animals was much lower than the test dose the authors did not comment on the possibility that isoflavones other than genistein were likely to have been present in the soybean fraction of this diet. Specifically, the changes observed in control and treated-dogs included altered ovarian size, ovarian cysts, thickened oviducts and uterine nodules, which have been reported as consequences of isoflavone-induced activity in other studies (Awoniyi *et al.* 1998; Gallo *et al.* 1999; Masutomi *et al.* 2003). Furthermore, the small sample size in the study complicates interpretation of the significance of a finding of 1 out of 2 dogs (50%) in one test group exhibiting altered reproductive parameters (McClain *et al.* 2005).

The most common flaw observed in uterotrophic studies is the failure to synchronise or standardise the timing of organ collection according to stage of oestrous cycle (the exceptions being Awoniyi *et al.* 1998; Brown *et al.* 1998; Diel *et al.* 2006). This is especially surprising in rodent studies, given the ease with which vaginal cytology can be utilised to determine stage of oestrous in these species. Moreover, the potential for large intra-group variation due simply to normal responses to the endogenous endocrine environment would not be controlled without such synchronisation. The common use of the immature rodent or ovariectomised (OVX) rodent model when assessing reproductive function or interference is also of concern. The role of endogenous oestrogen in modulating isoflavone effects is discussed in Section 1.4.4.3, whilst studies limiting the monitoring of reproductive parameters to pre- or peri-pubertal time periods risk missing significant perturbations later in an animal's life.

However, despite apparent discrepancies in the literature, isoflavones generally appear to be capable of affecting reproductive tissue structure and/or organisation, mimic oestradiol (E_2), and cause an increase in organ weight. The clinical significance of this is unclear

since uterotrophic effects are not consistently associated with impaired fertility or fecundity. However, due to the complex nature of reproductive physiology, activity at the tissue or cellular level indicates the potential for biological consequences in the organ, or body as a whole. Therefore, monitoring multiple biological parameters is necessary to determine isoflavone potential in the context of the whole animal.

1.5.2.3. *Oestrous cycle perturbations*

Isoflavone exposure has caused perturbations to human reproductive cycles, including lengthening, shortening and the introduction of irregularity in the menstrual cycle (Cassidy *et al.* 1994; Watanabe *et al.* 2000). The observation of any association between these changes and alterations in luteinising hormone (LH), follicle-stimulating hormone (FSH), oestrogen, sex hormone binding globulin, androstenedione, and/or testosterone has varied between study conditions (Cassidy *et al.* 1994; Watanabe *et al.* 2000).

The reported effect of isoflavone exposure on vaginal cytology and non-human cyclicity is also variable. No effect has been reported in some studies (Levy *et al.* 1995; Cline *et al.* 1996; Tansey *et al.* 1998; Duncan *et al.* 1999ab; Nikander *et al.* 2005; Nikaido *et al.* 2005; Manonai *et al.* 2006; McClain *et al.* 2006b; Wood *et al.* 2006ab), while other studies have shown significant keratinisation of vaginal smears (representative of a semi-persistent oestrus state), as well as inflammation and dyskeratosis in both a time- and dose-dependent manner (Wilcox *et al.* 1990; Whitten and Naftolin 1992; Whitten *et al.* 1993 and 1995; Gallo *et al.* 1999; Lewis *et al.* 2003; Uesugi *et al.* 2003; Malaivijitnond *et al.* 2004; Hidalgo *et al.* 2005; Malaivijitnond *et al.* 2006). Oestrous cycle irregularity (typically involving prolonged oestrus) has also been reported (Awoniyi *et al.* 1998; Gallo *et al.* 1999; Nagao *et al.* 2001; Kouki *et al.* 2003; Nikaido *et al.* 2004; Jefferson *et al.* 2005).

Cycle irregularities may occur as a result of isoflavone-induced negative feedback on follicle stimulating hormone (FSH) and luteinising hormone (LH), although direct oestrogenic effects on the vagina may also be possible (Gallo *et al.* 1999). For example, genistein is known to induce the formation of secondary interstitial cells from theca cells of atretic follicles and their subsequent synthesis of androgens may contribute to a deranged ovarian cyclicity (Awoniyi *et al.* 1998). Reports of altered vaginal cytology without concurrent perturbations in hormone concentrations suggest that isoflavones may have direct effects on the vaginal epithelium, or that vaginal cells demonstrate heightened sensitivity to exogenous oestrogenic compounds. Conversely, studies reporting no effect on cytological or hormonal parameters may reflect the ability of homeostatic mechanisms to negate the effects of dietary isoflavones on circulating hormone concentrations. This in turn may dampen cytological changes. It follows that acute exposure to supra-physiological doses may demonstrate short-term responses prior to a return to baseline, whilst chronic exposure to more physiologically-relevant doses may not yield detectable changes (e.g. McClain *et al.* 2006b). Whether direct or indirect, any activity of isoflavones to modulate reproductive cycles has important consequences for life-time fertility and fecundity, either by prolonging or shortening physiological readiness for conception.

1.5.2.4. *Hormonal influence*

The sex steroids play an integral part in determining the reproductive status of an animal, and are involved in a complex series of feedback controls. Any influence on hormone concentrations or feedback mechanisms has the potential to influence reproductive cycles, conception opportunities and tissue preparation for pregnancy. The effects of soy and purified isoflavones on gonadotrophins (LH and FSH), progesterone (P_4), sex hormone binding globulin (SHBG), E_2, oestrone (E_1), and testosterone are variable within the literature.

A number of studies have reported a lack of effect on certain hormone profiles (Cassidy *et al.* 1994; Anthony *et al.* 1996; Awoniyi *et al.* 1998; Uesugi *et al.* 2003; Malaivijitnond *et al.* 2004; Hidalgo *et al.* 2005; Nettleton *et al.* 2005a). On the other hand, some studies have demonstrated decreased LH (Cassidy *et al.* 1994 and 1998; Duncan *et al.* 1999a; Bennetau-Pelissero *et al.* 2001; Wu *et al.* 2005), E_2, and P_4 concentrations (Awoniyi *et al.* 1998; Cassidy *et al.* 1998; Duncan *et al.* 1999ab; Badger *et al.* 2001; Bennetau-Pelissero *et al.* 2001; Wu *et al.* 2005), as well as dehydroepiandrosterone sulphate concentrations (Cassidy *et al.* 1998). Additionally, increased sex hormone binding globulin and a decreased E_2:SHBG ratio were reported by others (Duncan *et al.* 1999b; Pino *et al.* 2000), which may indirectly alter the oestrogenic exposure of reproductive tract tissues. In contrast, modulations of morphological reproductive parameters have been reported to occur in the absence of hormonal changes (Malaivijitnond *et al.* 2004; Hidalgo *et al.* 2005), suggesting isoflavones may function locally at the tissue level.

The highly variable diurnal fluctuations in steroid hormones may render them insensitive and relatively unreliable biomarkers for isoflavone action. However, the variable duration of exposure, interindividual variation in metabolic capacity and the form/route of isoflavone administration are also likely to complicate comparisons between studies. Effects on steroid hormone concentrations must be considered in conjunction with histological changes in order to determine if changes are elicited via perturbation at the hypothalamus-pituitary or gonadal level. Ultimately though, if cycles and/or reproductive tissues are unaffected, any alteration in hormone concentrations is of dubious clinical relevance.

1.5.2.5. *Reproductive development*

Development of the various components of the reproductive system occurs during critical windows in an animal's life. Exposure to compounds with oestrogenic potential during these periods may alter the normal endocrine environment. However, the effect of isoflavone consumption on the development of reproductive parameters appears to vary

according to species, as well as with the type of isoflavone consumed, period of exposure and duration of exposure.

The majority of the literature in rodents points towards the ability of isoflavones to accelerate puberty onset (Whitten and Naftolin 1992; Hilakivi-Clarke *et al.* 1998; Casanova *et al.* 1999; Gallo *et al.* 1999; Badger *et al.* 2001; Delclos *et al.* 2001; Kouki *et al.* 2003; Lewis *et al.* 2003; Nikaido *et al.* 2004 and 2005; Takashima-Sasaki *et al.* 2006), although one study also demonstrated a delay in puberty (Levy *et al.* 1995). Altered development of the neuroendocrine system has occurred after isoflavone exposure (Faber and Hughes 1993; Whitten *et al.* 1993; Levy *et al.* 1995; Zhao *et al.* 2004), while modulations in ovarian function, cyclicity and aberrant sexual differentiation of the hypothalamus and pituitary cells have also been reported (Polkowska *et al.* 2004; Wójcik-Gładysz *et al.* 2005; Patisaul *et al.* 2006). Contrastingly, there is also strong evidence to support a lack of developmental effects following isoflavone exposure (Awoniyi *et al.* 1998; Strom *et al.* 2001: Nagao *et al.* 2001; Kang *et al.* 2002; Hughes *et al.* 2004; Pace *et al.* 2004; Naciff *et al.* 2004; Ryökkynen *et al.* 2005; Pace *et al.* 2006; Wilhelms *et al.* 2006).

The pre-pubescent development of the reproductive system is critical in determining life-time reproductive capacity and success. Interference during this developmental stage is often irreversible and, depending on the nature and extent of perturbation, may result in altered reproduction later in life. As such, isoflavone-induced changes in normal reproductive development, which may not be detected for significant periods of time following isoflavone exposure, have great potential for the modulation of reproductive parameters in adulthood. Yet, as with other factors discussed for isoflavone bioavailability and reproductive effects, the consequences of isoflavone intake on reproductive development are far from predictable.

1.5.2.6. *Molecular and biochemical activity*

Examination of the ability of endocrine disruptors to regulate oestrogen-sensitive genes is thought to provide a reliable method for the characterisation of *in vivo* oestrogenicity (Diel *et al.* 2000). Indeed isoflavones have produced changes in gene expression, cell signalling and other molecular level effects in the absence of perturbations in other reproductive endpoints (Diel *et al.* 2000; Brown and Setchell 2001). Changes have also been observed following isoflavone exposure that was not predicted from studies with E_2 (Adachi *et al.* 2005).

Gene transcription, apoptosis, cell-cycle regulation, cellular proliferation, anti-oxidant pathways, signal transduction pathways as well as genes concerned with oncogenesis have all shown sensitivity to genistein exposure (Konstantakopoulos *et al.* 2006). However, these *in vitro* effects may not translate to detectable effects *in vivo*, especially considering the high potential for other interacting and confounding variables in an *in vivo* environment. Yet, although genistein concentrations at the lower end of the physiological range may not exert significant *in vitro* effects on gene transcription, it is important to consider that the duration of *in vivo* exposure may be longer than that of *in vitro* studies (Konstantakopoulos *et al.* 2006). Additionally, *in vitro* concentrations are based on circulating isoflavone concentrations in plasma and serum, which may not reflect concentrations occurring at the tissue level where accumulation may occur, especially in target tissues (Konstantakopoulos *et al.* 2006; Section 1.3.6).

Overall, the clinical significance/relevance of altered gene expression in the absence of histological changes is unclear and requires long term studies to fully elucidate. The complexities of animal physiology suggest that findings of a perturbation at the genetic or molecular level should be interpreted with caution.

1.5.2.6.a. *Sex steroid receptor and oestrogen-responsive gene regulation*

Modulation of ERs and oestrogen-response element (ERE)-dependent gene expression by genistein is one of the primary mechanisms implicated in the reproductive potency of genistein and other isoflavones (Diel *et al.* 2000; Cotroneo *et al.* 2001; Lee *et al.* 2004; Staar *et al.* 2005; Diel *et al.* 2006; Seo *et al.* 2006; Chrzan and Bradford 2007). Interactions between endogenous hormones and their receptors are critical in the control and activation of reproductive processes, and form the basis of endogenous hormone control over reproduction. Therefore, by binding to sex steroid receptors, or altering their expression, isoflavones may modify the activity of endogenous hormones and influence normal reproductive functions.

Changes (up-regulation or down-regulation) in receptor expression have been shown to occur in an ovariectomised (OVX) animal model in which circulating hormones were unaffected by isoflavones (Cotroneo *et al.* 2001). Therefore, induction of receptor expression is unlikely to occur as a result of changes in circulating steroid hormone concentrations, indicating direct gene expression effects. However, effects are not uni-directional and both down-regulation (Sathyamoorthy and Wang 1997; Cotroneo *et al.* 2001 and 2005; Diel *et al.* 2005; Wang *et al.* 2005; Padilla-Banks *et al.* 2006; Seo *et al.* 2006), and up-regulation (Jefferson *et al.* 2002a; Lee *et al.* 2004; Petroff *et al.* 2005; Staar *et al.* 2005) of the ER has been reported.

The effect of isoflavones on the two subtypes of the ER (α and β), varies according to species, tissue, cell system and laboratory. Some studies have reported a lack of effect on ERβ, but significant effect on ERα (Cotroneo *et al.* 2001; Lee *et al.* 2004; Petroff *et al.* 2005), while others have reported a significant effect on both (Patisaul *et al.* 2001; Ren *et al.* 2001; Staar *et al.* 2005; Cappelletti *et al.* 2006; Padilla-Banks *et al.* 2006; Takashima-Sasaki *et al.* 2006; Chrzan and Bradford 2007). However, ERβ ligands (such as genistein) may regulate some oestrogen-responsive components via the ERα pathways, without any modulation of ERβ expression (Lee *et al.* 2004). Furthermore, genistein is capable of modifying the relative distribution of ERα and ERβ subtypes, but the effect

changes according to the cell type investigated (Cotroneo *et al.* 2001; Cappelletti *et al.* 2006).

1.5.2.6.b. *Growth factors, cellular proliferation and enzyme inhibition*

The reported isoflavone-induced changes in cellular or tissue growth, development and/or hormone profiles may occur as indirect consequences of isoflavone interference in critical enzyme or growth factor pathways. Studies have shown that growth factors are influenced by isoflavones, each with distinct and variable suppressive or proliferative growth effects. Suppression of cellular growth by isoflavones may be mediated by isoflavone-induced inhibition of proliferating growth factors (e.g. Epidermal Growth Factor, EGF), and/or by production and expression of growth-inhibiting factors (e.g. Transforming Growth Factor β (TGF-β) (Barnes *et al.* 2000). In contrast, the uterotrophic effects of isoflavones may occur as a result of action to increase uterine stromal EGF, reduce uterine and blood vessel EGF receptor and/or decrease TGFα in the glandular and luminal uterine epithelium (Brown and Lamartiniere 2000; Cotroneo *et al.* 2001). The reported ability of isoflavones to protect against certain inflammatory responses may be the result of their inhibition of cytokines such as Tumour Necrosis Factor α (TNF-α) (Kang *et al.* 2005).

Isoflavones are known to inhibit protein phosphorylation, and this has been reported as their mechanism of action against the EGF receptor (Peterson and Barnes 1993; Barnes *et al.* 2000). Interestingly, daidzein and equol are both capable of inducing phosphorylation to a 2-fold greater extent than achieved by E_2 (Totta *et al.* 2005). Nonetheless, protein phosphorylation is not the universal mechanism by which isoflavones exert their influence on growth factors (Barnes *et al.* 2000). Transcriptional changes rather than direct activity on tyrosine kinase activity have also been reported, whilst certain tyrosine kinases actually appear unresponsive to isoflavones (Brown *et al.* 1998; Barnes *et al.* 2000).

Isoflavones are also capable of modulating the production of other enzyme systems, such as aromatase (Adlercreutz *et al.* 1993; Edmunds *et al.* 2005; Lacey *et al.* 2005), which has consequences for oestrogen metabolism. However, inhibition of the enzymatic conversion of pregnalone to P_4, and androstenedione to E_2 following co-incubation of genistein or daidzein with human granulosa-luteal cells were only achievable *in vitro* at concentrations ≥ 10 μM (Lacey *et al.* 2005). This suggests that some enzyme-mediated effects of isoflavones are unlikely to occur *in vivo.*

Markers for cellular proliferation (e.g. proliferative cellular nuclear antigen, PCNA), and apoptosis are reported to be unaffected by soy consumption (Tansey *et al.* 1998; Eason *et al.* 2005). However, purified genistein appeared to be capable of eliciting significant changes in proliferative endpoints, both *in vivo* and *in vitro* at physiological concentrations (Eason *et al.* 2005; Ford *et al.* 2006; García-Pérez *et al.* 2006). These changes show a concentration-dependent response, with E_2-induced proliferation enhanced by genistein at low concentrations (< 10 μM), but ERE and cellular proliferation suppressed at higher concentrations (Wang *et al.* 1996; Po *et al.* 2002). This high-concentration suppressive effect was suggested to be due to cytotoxic events, since inhibition was demonstrated in ER-negative cell lines (Wang *et al.* 1996). Daidzein has also been suggested to elicit its effects in this way (Lehmann *et al.* 2005). However, again caution must be heeded when interpreting findings that cannot be replicated in studies utilising natural dietary forms of genistein.

1.5.2.7. *Fertility and fecundity*

Ultimately, any study investigating agents with the potential to influence the reproductive system are concerned with the outcome of this activity on fertility and fecundity. To this end, a number of studies have demonstrated impaired fertility and fecundity following isoflavone exposure. Perhaps the most frequently cited isoflavone-induced phenomenon is that of "clover-disease" in sheep. Phytoestrogen consumption (via clover pasture) resulted in cumulative and persistent infertility during two years of exposure, which

persisted beyond the exposure period (Bennetts *et al.* 1946; McDonald 1995). However, effects were also observed in sheep exposed to clover pasture for shorter periods, whereby sperm and ovum transport were impeded, ovulation rates were lowered, oestrus incidence was decreased, and fertilization was impaired (McDonald 1995).

A lowered fertility rate in cattle consuming isoflavones has also been reported and the potential mechanism for this finding is thought to be the isoflavone-induced suppression of luteolytic prostaglandin F ($PGF_{2\alpha}$) production (Woclawek-Potocka *et al.* 2005ab). Poor gamete quality, lowered fertilization rates and reduced delivery rates have also been linked with genistein treatment in other species (Gallo *et al.* 1999: Bennetau-Pelissero *et al.* 2001; Delclos *et al.* 2001; Nagao *et al.* 2001; Becker *et al.* 2005; Jefferson *et al.* 2005; Padilla-Banks *et al.* 2006). Furthermore, early reproductive senescence, interference with implantation, perturbation within the hypothalamic-pituitary-gonadal axis perturbation, and reduced post-implantation viability or increased resorption are also known to be associated with isoflavone exposure (Gallo *et al.* 1999; Delclos *et al.* 2001; Jefferson *et al.* 2005; Padilla-Banks *et al.* 2005; Wu *et al.* 2005).

Sexual behaviour may also be affected. The demonstration of sexual receptivity (lordosis quotients) was reduced after exposure to phytoestrogens in rodents (Patisaul *et al.* 2001; Kouki *et al.* 2003; Kudwa *et al.* 2007). Even in the absence of physiological impairments in the reproductive system, behavioural changes resulting in a sub-optimal sexual response can drastically reduce fertility.

These findings of altered fertility or fecundity in association with isoflavone exposure may arise due to activity at the cellular, tissue, or hormonal level, and influence within the neurological programming may also be important. The mechanisms of such abrogated fertility and fecundity are important in determining severity or permanence of effects.

Conversely, isoflavone consumption has also been shown to result in no detectable changes in fecundity parameters in a number of ovine, mustelid or rodent fertility studies (Levy *et al.* 1995; Gallo *et al.* 1999; Lamartiniere *et al.* 1998; Badger *et al.* 2001; Pace *et al.* 2004; Ryökkynen *et al.* 2005; Pace *et al.* 2006; Ryökkynen *et al.* 2006). Gestational length, number of offspring, proportion of live young and sex ratio were all reported to be unaffected by consumption of isoflavones (Lamartiniere *et al.* 1998; Gallo *et al.* 1999; Delclos *et al.* 2001; Kang *et al.* 2002; Becker *et al.* 2005). Indeed, one study has actually reported increased egg-laying capacity and egg mass in ducks exposed to dietary daidzein (Zhao *et al.* 2004), and an equal incidence of mating and conception between treatment and control groups have been reported in rodents (Jefferson *et al.* 2005).

1.6. Effects of Isoflavones in the Hepatic System of Non-Felid Mammals

1.6.1. Background

Although the classical targets of oestrogen activity are tissues containing significant ER concentrations (e.g. the organs of the reproductive tract), the liver is also a target of oestrogen activity (Diel *et al.* 2002). To this end, isoflavones have been associated with a number of perturbations in the hepatic system.

1.6.2. Hepatic Hypertrophy and Hepatopathy

Both genistein and daidzein have been associated with hypertrophic effects in the liver (Banz *et al.* 2004; McClain *et al.* 2006b). However, studies with soy protein isolate have produced divergent results, whereby some studies have reported no effect on liver weight in female rats, but others have reported reduced liver weights in males (Peluso *et al.* 2000; Huang *et al.* 2005; Tachibana *et al.* 2005). Likewise, mild hepatoxicity was only demonstrated following exposure to 500 mg/kg BW, and these changes were reversible, suggesting that normal dietary exposure is unlikely to pose a risk to hepatic health (McClain *et al.* 2006b).

It is feasible that gender-based differences in isoflavone accumulation in the liver are important in determining these differences between studies (see Section 1.3.6.2). However, chronic exposure to doses as high as 500 mg genistein/kg BW failed to demonstrate permanent changes in rat liver weight, and relative liver weight had normalised following an 8 week recovery period (McClain *et al.* 2006b). The lack of permanence of effects in this regards indicates that the liver may be responding directly

to the presence of isoflavones rather than enduring fundamental histological changes. Although this may be used to argue against detrimental effects of isoflavones in the hepatic system, life-time exposed animals may suffer secondary consequences of a persistently increased hepatic mass.

1.6.3. Hepatic Steroid Metabolism

Hepatic glucuronidation of oestrogen metabolites permits their excretion into the bile (Pfeiffer *et al.* 2005) and functions to maintain these hormones in an efficient enterohepatic recirculation (Adlercreutz *et al.* 1987). Genistein and daidzein have contrasting effects on the metabolic clearance of E_2 in the human liver, with daidzein known to enhance E_2 excretion through the up-regulation of glucuronidation, while genistein has an inhibitory effect on glucuronidation (Pfeiffer *et al.* 2005). Neither isoflavone was effective in modulating E_2-glucuronide formation, so the UGT2B7 isoform responsible for this type of conjugation is apparently unaffected by isoflavones (Pfeiffer *et al.* 2005). Interestingly, co-administered genistein (as would occur following dietary soy intake), may counteract daidzein activity or even produce antagonistic effects (Pfeiffer *et al.* 2005). Interspecific differences also exist, whereby no stimulation of E_2 glucuronidation was observed in rat liver microsomes exposed to isoflavones (Pfeiffer *et al.* 2005). Hepatic testosterone hydroxylation is also known to be modulated by genistein or soy protein (Laurenzana *et al.* 2002; Ronis *et al.* 2004 and 2006a). However, one study has failed to detect any influence on the metabolism of this sex steroid (Badger *et al.* 2001).

Isoflavones may indirectly influence the reproductive tract by altering hepatic steroid concentration and/or activity. Additionally, isoflavones also appear to have an inhibitory effect on the hepatic conjugation of other compounds, potentially reducing the detoxifying capacity of the liver towards certain compounds (Jäger *et al.* 1997; Lucas *et al.* 2003). The mechanism by which genistein inhibits hepatic conjugation is thought to be primarily competitive in nature and independent of protein tyrosine kinase inhibition

(Jäger *et al.* 1997; Lucas *et al.* 2003). Potential also exists for counter-transport of genistein (or its glucuronides) into hepatic tissue. This could replace intracellular concentrations of other hepatically generated conjugates, and further increase hepatic and systemic exposure to these latter compounds (Lucas *et al.* 2003). Enhanced exposure to these relocated compounds may cause physiological responses in their own right, which may not initially appear attributable to isoflavone activity.

1.7. Potential for Influence of Isoflavones in Domestic Cat

The reproductive and hepatic consequences of isoflavone exposure in mammalian species remain controversial. However, overall the literature indicates that dietary isoflavones are capable of exerting significant effects in a number of parameters, albeit with various influencing factors and inter-specific, tissue-, gender- and age-related differences. Therefore, as a mammal known to consume dietary isoflavones, the cat and cheetah (see Section 1.2.1.2) may also exhibit physiological responses to isoflavone exposure. However, an understanding of their capacity to absorb, metabolise and excrete isoflavones is required before biological activity can be predicted.

1.7.1. Isoflavone Absorption and Excretion in Felids

The first indication that dietary isoflavones may be absorbed by a felid species was provided by Setchell *et al.* (1987b). Cheetahs consuming a diet containing soy protein produced urine containing a number of unidentified phenol-like compounds in the unconjugated steroid fraction (Setchell *et al.* 1987b). These compounds were thought to represent metabolites of genistein and daidzein, which were found in the diet.

More recent research has shown that isoflavones are excreted in the urine of domestic cats, and that consumption of a low isoflavone diet results in significant differences in urinary genistein *versus* daidzein urinary excretion in this species (White *et al.* 2004). All cats consuming either a low- or high-isoflavone diet excreted detectable concentrations of genistein, whilst daidzein was detectable in the urine of only two of 18 cats in the low-isoflavone group, and 17 of 18 cats in the high-isoflavone group (White *et al.* 2004). The finding of lower urinary concentration of daidzein relative to genistein in cats is in contrast to that observed in humans and rodents, where genistein recovery has consistently been shown to be lower compared to daidzein. However, only single point-

in-time urinary samples were collected from cats in the study of White *et al.* (2004) and are hence unlikely to represent total urinary excretion profiles.

1.7.2. Isoflavone Metabolism in Felids

Although no studies have been conducted specifically to determine the capacity of feline species to metabolise dietary isoflavones, Brown *et al.* (1994) offered some evidence that domestic cats are capable of converting daidzein into equol through their incidental finding of this metabolite in domestic cat faeces.

Cave *et al.* (2007a) quantified the relative proportions of unbound and conjugated genistein in cat plasma. This study determined that cats possess significant capacity for the conjugation of genistein, although the specific metabolites were not identified (Cave *et al.* 2007a). By assessing the plasma ratio of total-to-unbound genistein concentration in cats, Cave *et al.* (2007a) demonstrated a greater ratio following oral compared to I/V exposure at all time points, indicating a role for enteric and/or hepatic metabolism (Cave *et al.* 2007a). Although the cat is typically assumed to be deficient in its capacity for glucuronidation, showing only limited glucuronidation activity towards the majority of xenobiotics tested (see Section 1.7.2.1.), conjugation of isoflavones by cats *in vivo* is reported to be comparable to rats, mice and humans (Cave *et al.* 2007a). This contradicts the hypothesis of Setchell *et al.* (1987ab), that felids may be more susceptible to the biological effects of isoflavones due to their reduced aptitude for hepatic glucuronidation.

1.7.2.1. *Steroid and Xenobiotic Metabolism in Felids*

Given the dearth of information pertaining to isoflavone metabolism in felids, it is pertinent to explore the metabolic processes of felids towards other compounds with structural resemblance to isoflavones.

A couple of studies (Setchell *et al.* 1987a; Court and Freeman 2002) have speculated that the reduced capacity of cats to conjugate compounds with glucuronic acid may render them unable to metabolise isoflavones. Indeed conjugation with glucuronic acid has been shown to be defective in the cat against a large number of substrates, and was the primary mechanism identified for acetaminophen poisoning in this species (Larson 1963; Capel *et al.* 1972; Miller *et al.* 1973; Capel *et al.* 1974; French *et al.* 1974; Wong 1976; Schmoldt *et al.* 1982; Court and Greenblatt 2000; Krishnaswamy *et al.* 2004).

The family of enzymes responsible for many detoxification pathways in other mammals (UDP-glucuronosyltransferase, UGT) are actually expressed in the cat liver (Court and Greenblatt 1997 and 2000). However, the range of isoforms is considerably limited and UGT activity is significantly lower in the cat than other mammalian species (Hietanen and Vaino 1973; Schmoldt *et al.* 1982; Court and Greenblatt 1997 and 2000). Furthermore, felids lack one of the cytosolic arylamine *N*-acetyltransferase (NAT) enzymes known to be important in xenobiotic metabolism (Trepanier *et al.* 1998). Although felids retain NAT1 (expressed in the liver, colon and bladder, but not the duodenum), the poor catalytic activity of this enzyme, compared to the absent NAT2, is thought to confer a lower capacity for metabolism of certain xenobiotics via this route (Trepanier *et al.* 1998).

However, there is a high degree of overlap in function between UGT isoforms, and substantial redundancy has been indicated (Shangari *et al.* 2005). The few UGT isoforms that are functional and expressed in the cat liver, have been suggested to play a role in the metabolism of xenobiotics (Court and Greenblatt 2000; Krishnaswamy *et al.* 2004; Shangari *et al.* 2005). Indeed, evidence for at least partial glucuronidation of some compounds by cats is found in the literature (Capel *et al.* 1972; Miller *et al.* 1973; Capel *et al.* 1974; Schmoldt *et al.* 1982; Hassell *et al.* 1984; Krishnaswamy *et al.* 2004). Certain substrates were preferentially glucuronidated (Capel *et al.* 1974), occasionally even to a similar or greater extent than achieved by humans (Hassell *et al.* 1984; Court and Greenblatt 2000). The glucuronidation of endogenously generated compounds such

as bilirubin, steroids and thyroid hormones in the cat has been shown to be similar to other species (Court and Greenblatt 2000), possibly due to the expression of UGT1A1 in cat liver, which is primarily responsible for these reactions (Burchell *et al.* 2005). These findings, therefore, indicate that the possibility exists for hepatic glucuronidation of isoflavones by cats.

Moreover, glucuronidation is not the only metabolic pathway available for isoflavone conjugation. Sulphotransferases (SULTs) are ubiquitous in mammalian cells, but highly concentrated in the liver, where they are responsible for conjugation of hydrophobic molecules or protein amino acids (Shangari *et al.* 2005). It is thought that SULTs work in a complementary manner with UGTs, providing an efficient "back-up" for conjugating molecules that fall below the peak threshold for glucuronidating enzyme activity (Shangari *et al.* 2005). Sulphation was the predominant pathway for systemic metabolism of a number of xenobiotics in felids, and the conjugation of phenol in rat livers was approximately equal between sulphate and glucuronic acid (Capel *et al.* 1972; Miller *et al.* 1973; Capel *et al.* 1974; French *et al.* 1974; Wong 1976). Hence, isoflavones escaping glucuronidation in the cat liver may still be conjugated to a sulphate moiety, and subsequently de-activated and excreted in this form.

Additionally, the focus on hepatic metabolism in the cat may be misleading since it is now understood that the intestinal mucosa plays a more important role in isoflavone metabolism (Cave *et al.* 2007a; see Section 1.3.2.2). Moreover, conjugation of phenol to sulphates by cat intestinal tissue has been indicated in some studies (Capel *et al.* 1972). However, sulphate conjugation was shown to be readily saturable in some studies and the toxic effects of phenols in felids explained by the presence of large numbers of unconjugated and oxidated phenol products (Larson 1963; Miller *et al.* 1973). Consequently, although felid intestinal enzymes may be capable of conjugating certain phenols, perhaps including isoflavones, a significant fraction may still escape this first-pass metabolism. If this fraction were also to escape hepatic conjugation, significant physiological effects could be predicted from the high circulating concentrations of these

unbound compounds. However, the relative importance of enteric and hepatic conjugation, as well as the forms of metabolites produced, has yet to be determined in the cat.

1.7.3. Pharmacokinetics and Bioavailability

The first comprehensive study of the bioavailability of an isoflavone (genistein) in domestic cats has only recently been published (Cave *et al.* 2007a). These authors utilised a supra-physiological oral dose (100 mg/kg BW), which would be unachievable from consumption of any investigated commercial feline diet (Court and Freeman 2004; Bell *et al.* 2006). However, the experiment provides valuable insight into the pharmacokinetics of genistein in the cat. Cave *et al.* (2007a) included frequent sampling points, both I/V and oral administrations, and an extended collection period such that their calculation of area under the curve (AUC) and terminal elimination half life can be considered an accurate reflection of genistein bioavailability, at this dose.

Genistein was quantifiable in serum up until 54 h following ingestion, but remained detectable in two of the six cats until 72 h post-dose (Cave *et al.* 2007a), emphasising the need for extended sampling periods in bioavailability studies. The distinction was also made between conjugated and unbound forms of genistein in circulation (Cave *et al.* 2007a), although no further identification of specific metabolites was reported. Overall, the oral bioavailability of unbound genistein was poor (1.4%), whilst conjugated genistein (reported as unbound equivalents) had a greater apparent bioavailability (29.9%) (Cave *et al.* 2007a). This effect has also been suggested in humans (Setchell *et al.* 2001), but pharmacokinetics were not computed for unbound genistein and doses utilised were considerably lower than used in cats (BW of human participants were unreported, but an estimate of 60 kg gives a dose of 0.8 mg/kg BW based on a 50 mg administration) .

The difference between unbound and conjugated genistein bioavailability in cats emphasises the need to clarify the various isoflavone forms found in circulation. The reporting of bioavailability for total genistein equivalents alone would have resulted in considerable overestimation of bioavailability, in this case. The reason for such low oral genistein bioavailability in cats is thought to be a combination of poor absorption and efficient intestinal or hepatic first pass metabolism (Cave *et al.* 2007a).

It should be noted that Cave *et al.* (2007a) utilised food-deprived cats, so the kinetics of isoflavones following dietary intake may differ in cats in which food is present in the GIT. Administration of doses representative of dietary contents, and the administration of isoflavones within a dietary matrix would be necessary to fully elucidate the pharmacokinetics and bioavailability of isoflavones in domestic cats and cheetahs.

1.7.4. Isoflavone induced changes in felid physiology

One study has reported the possible association of endocrinological changes and isoflavone intake in the domestic cat (White *et al.* 2004). Significant changes (although modest, and mostly within the normal range of variation) in thyroid hormone concentrations were reported when cats consumed a soy-containing diet, compared to periods of soy-free diet intake (White *et al.* 2004). The isoflavone exposure of these cats was not reported but can be estimated from the reported dietary content to be in the range of 3 mg/kg BW for both genistein and daidzein. This dose is within the range reported for commercially-prepared diets (Court and Freeman 2004; Bell *et al.* 2006), and supports the potential for isoflavone-induced reproductive perturbation in this species following dietary exposure.

Isoflavones have also been shown to affect physiological processes in another felid species. The histological abnormalities observed in captive cheetahs consuming diets containing soy protein are reported to be reminiscent of those detected in sheep suffering

from "clover disease" (Setchell *et al.* 1987ab). Uterine lesions observed in cheetahs were characterised by cystic endometria, myometrial and endometrial fibrosis, all of which are thought to potentially interfere with normal implantation and nutrition of the fertilised egg (Setchell *et al.* 1987a). Setchell *et al.* (1987a) also suggest that the acyclicity and sexual unresponsiveness observed in captive cheetahs may be indicative of isoflavone-interference at the hypothalamic-pituitary level. To this end, soy isoflavones have demonstrated the ability to alter reproductive cycles in a number of species (see Section 1.5.2.3).

However, the circumstantial data presented by Setchell *et al.* (1987ab) which is used to suggest that isoflavones may be aetiological in these reproductive parameters requires validation with an appropriate animal model (Setchell *et al.* 1987b). Despite being repeatedly cited as having determined a link between dietary isoflavones and infertility in the captive cheetah, the study by Setchell *et al.* (1987) is anecdotal and speculative. It does not include epidemiological evidence to support the association since a multi-institutional survey was not conducted to link reproductive parameters and isoflavone intake.

The facilities listed in the International Cheetah Studbook as most successfully breeding cheetahs were typically dedicated breeding centres (Marker 2005), holding large populations of cheetahs and employing management strategies dedicated to the breeding process (pers. obs.). In contrast, the majority of North American facilities holding cheetah house them as part of a larger zoological collection, primarily aimed at public display and education purposes (pers. obs.). Hence, the comparison made by Setchell *et al.* (1987a) between North American facilities and a South African breeding centre is biased by the divergent management regimes and breeding efforts. Diet is therefore likely to be one of many other husbandry factors that may explain the divergent breeding success.

A further hypothesis of these authors was that isoflavones may be involved in veno-occlusive disease (VOD), also known as hepatic fibrosis, in captive cheetahs (Setchell *et al.* 1987ab; Gosselin *et al.* 1988), which is reported to cause significant mortalities in the captive cheetah population (Munson *et al.* 2005). Reasoning for their hypothesis was similar to that of their reproductive influence hypothesis, in that the reported occurrence of VOD in North American cheetahs was nearly 60%, compared to the apparent lack of the disease in cheetahs at one South African facility with low dietary exposure to isoflavones (Setchell *et al.* 1987a; Gosselin *et al.* 1988). However, 103 liver sections from cheetahs held in North American zoos were compared to only seven sections from cheetahs held in the South African facility. This limited data, coupled with observations of changes in liver chemistry and histology following a change in diet from a soy-containing diet, to one devoid of soy, were used to support their hypothesis (Setchell *et al.* 1987a).

Hepatic fibrosis is a dynamic process involving a complex of processes, initiated either extra- or intra-hepatically (Center 2004). Indeed, the non-specific mechanisms of hepatic fibrosis which initially occur in response to hepatic injury can in turn themselves become inhibitory of normal hepatic functioning (Center 2004). Therefore, the multitude of hepatic insults which may culminate in hepatic fibrosis must be considered before aetiological responsibility can be placed solely on dietary isoflavones in the case of VOD in cheetahs.

The soy-containing diet fed to the cheetahs was assayed (see Section 1.2.1.2) and determined to have oestrogenic potency via a rodent bioassay, utilising either partially or totally hydrolysed dietary extracts to distinguish between mammalian steroids and plant steroid content (Setchell *et al.* 1987a). Oestrogenic uterotrophic effects were only observed in extracts containing plant-derived steroids, and the isoflavones genistein and daidzein were thought to be responsible for this finding (Setchell *et al.* 1987a). It was concluded that removal of these oestrogens from cheetah diets may improve hepatic function and hepatic mitochondrial alteration (Setchell *et al.* 1987a).

Indeed, when such a dietary change was implemented in cheetahs, the authors report altered hepatic parameters including decreased total bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma glutamyl transferase (GGT) concentrations (Setchell *et al.* 1987a). In addition, hepatic mitochondria were altered in size and shape (towards a more normal shape), compared to the abnormal conformation observed in biopsies obtained during isoflavone consumption (Setchell *et al.* 1987a). A similar recovery of altered hepatic chemistry profiles was observed in rats following 52 weeks exposure to genistein (McClain *et al.* 2006b). However, hepatic effects in rats were only observed at doses exceeding 50 mg/kg BW, and were primarily restricted to the 500 mg/kg BW dose group. This level of exposure was dramatically higher than that of the cheetahs (1.25 mg/kg BW) involved in the study by Setchell *et al.* (1987a). Significant interspecific differences in absorption, metabolism, and hepatic response would therefore be required in order to support a role for dietary isoflavones as causative agents in the liver disease observed in captive cheetahs. In addition, fasting serum bile acids were excluded from the assessment panel utilised in the cheetah study (Setchell *et al.* 1987a). This is likely to have reduced the sensitivity of the testing panel since the sensitivity of assessments for hepatic function has been reported to be increased by up to 90% with a combination of ALT, total bilirubin and fasting bile acids in domestic cats (Jacob *et al.* 2002). It is important to consider that abnormalities in non-hepatic tissues may also result in test values outside the reference range (Roth-Johnson 2004). Thus, the specificity of serum hepatic enzymes in detecting certain hepatopathies is low (Webster 2005).

Importantly, the ability to evaluate liver function is restricted to the assessment of serum bilirubin, bile acids and ammonia, whereas ALT, aspartate aminotransferase (AST), alkaline phosphatase (ALP) and GGT are indicative only of hepatocellular injury, resulting in the leakage of these enzymes into circulation (Roth-Johnson 2004). Hormonal stimulation, systemic infections, hyperthyroidism, neoplasia, muscle necrosis, bone growth, fracture healing, drug metabolites, and cholestatic disease are among the

non-hepatic sources of increased serum concentration of these enzymes (Roth-Johnson 2004; Webster 2005). Therefore, it is possible that hepatic enzyme concentrations were altered in cheetahs for reasons other than the removal of dietary isoflavones from their diet. Alternatively, the metabolism of dietary isoflavones by the felid liver may stimulate the up-regulation of hepatic enzyme production, possibly without subsequent hepatic insult.

The study by Setchell *et al.* (1987ab) also includes a number of assumptions, which casts doubt on their conclusions. Firstly, their assumption of increased incidence of VOD in North American cheetahs due to dietary differences with a South African facility was not corroborated by dietary information for the cheetahs providing the evaluated liver sections. Instead, reliance was placed on anecdotal evidence of a greater consumption of soy containing diets in the North American population, compared to the meat-based, soy-free diets apparently used in the South African facility.

Secondly, the use of supplemented chicken meat as the soy-devoid trial diet for comparison with the horsemeat-based soy-containing diet introduced uncontrolled, confounding variables. The differing nutritional composition of the chicken and horsemeat diet is likely to induce hepatic changes independent of any oestrogenic compound-related effect. Horsemeat devoid of soy would have made a more appropriate comparison, although correction for the altered protein content would still have been necessary. A cross-over design may have further increased the power of the study, given the small number of animals ($n = 4$).

Additionally, the use of sub-cutaneous administration of extracts in the rodent bioassay limited the extent to which the results can be extrapolated to the findings in the cheetah. The sub-cutaneous route of administration by-passed major intestinal and hepatic metabolism, and would not expose the rodents to the same pharmacological pathway as the cheetahs. Likewise, a more appropriate model species could have been utilised for

the bioassay since interspecific variation in pharmacodynamics and metabolism between rodents and felids is possible, as are differences between felid and rodent physiological response to oestrogenic compounds. Furthermore, the inclusion of hepatic parameters in the rodent bioassay could have leant substantial weight to the conclusions drawn for cheetahs by Setchell *et al.* (1987a). Finally, the dose used in the bioassay was considerably greater (200 mg/kg BW) than administered to the cheetahs (1.25 mg/kg BW) by the diet. Extrapolation of such high exposures across unrelated species is questionable, although the bioassay was primarily aimed at determining oestrogenic potential of the diet.

In contrast to the conclusion of Setchell *et al.* (1987ab) and Gosselin *et al.* (1988), recent work has demonstrated a protective role for isoflavones against liver fibrosis and injury (Lee *et al.* 1995; Kang *et al.* 2001; Liu *et al.* 2002; Kuzu *et al.* 2007; Wong *et al.* 2007). Proliferation of hepatic stellate cells, which play a pivotal role in liver fibrosis and are often targets of therapeutic regimes (Liu *et al.* 2002), have been shown to be inhibited by genistein (Kang *et al.* 2001; Liu *et al.* 2002). Similarly, collagen synthesis and associated type I procollagen mRNA concentrations were suppressed by genistein, in a concentration-dependent manner *in vivo* (Kang *et al.* 2001; Liu *et al.* 2002).

Anti-carcinogenic effects of isoflavones have also been demonstrated, and include anti-inflammatory and anti-necrotic activity via prevention of lipid peroxidation, inhibition of cellular proliferation, and stimulation of antioxidation and apoptotic agents (Chodon *et al.* 2007; Kuzu *et al.* 2007). Following irradiation of cells, soy isoflavone exposure was reported to confer cells with the ability to maintain expression of DNA repair and stress response-genes, and other markers for cellular damage at close to normal concentrations (Song *et al.* 2006). The tumour protective effects of genistein in the liver may be further ameliorated by its ability to suppress lipopolysaccharide-induced increases in inflammatory cytokines and anti-inflammatory properties at concentrations as low as 0.19 μM (Zhao *et al.* 2006). Moreover, endogenous oestrogens (and therefore isoflavones if they were to mimic these hormones) are thought to play a role in inhibiting liver

mutagenesis, since intact animals exhibited lower mutation indexes than gonadectomised animals (Chen *et al.* 2005a).

However, genistein exposure did not demonstrate any modulatory effects in preventing chemically-induced genetic mutagenicity (Chen *et al.* 2005a). Instead, genistein may act as hepatotoxin in some situations (Lee *et al.* 1995), so the effects of isoflavones may be dependent on the underlying hepatic tissue status. In rats without concurrent hepatocarcinogenesis, isoflavones were carcinogenic in the liver (Lee *et al.* 1995). However, this effect may be inducer-specific, since no carcinogenic activity of genistein was observed in another study (Chodon *et al.* 2007) utilising a different cancer promoter. Variation in reported hepatic response to isoflavone exposure is likely to be due to a multitude of factors, including duration of exposure, timing of exposure, form of isoflavone, dietary interactions, as well as dose- and gender-specific effects.

In order to better assess the potential changes that may occur in the domestic cat reproductive tract following exogenous compounds possessing oestrogen agonistic or antagonistic properties, it is important to understand the effects of E_2 itself.

1.7.4.1. *Exogenous steroid administration to ovariectomised cats*

Removal of the ovaries results in significant depletion of circulating oestrogen concentrations and predictable histological changes within the reproductive tract of mammals. Exogenous oestrogen has been shown to be capable of reversing changes associated with ovariectomy in all mammalian species studied, including the domestic cat (Verhage and Brenner 1975; Bareither and Verhage 1980). Following E_2 exposure, mitotic activity was increased, and generalised hypertrophic and hyperplastic events have been observed in the OVX feline reproductive tract (Verhage and Brenner 1975; Bareither and Verhage 1980).

The removal of endogenous E_2 by gonadectomy resulted in the disappearance of P_4 receptors from all cells except the stromal fibroblasts, whilst ERα was still expressed in feline tissue (Li *et al.* 1992). Exogenous E_2 restored PR expression to concentrations equivalent to oestrus, and also increased the expression of ERα (Li *et al.* 1992). However, both endogenous and exogenous P_4 were antagonistic to the effects of E_2 in the luteal or exogenously-exposed OVX feline uteri (Li *et al.* 1992), and reduced ERα and PR concentrations to near OVX concentrations (Li *et al.* 1992). The major difference reported was in the persistence of ERα within glandular epithelium, and presence of both ERα and PR within stromal fibroblasts (Li *et al.* 1992). The distribution and expression of ERα and PR in the cat uterus therefore appears to be under E_2 and P_4 control (Li *et al.* 1992) and significant variation in expression and distribution could be expected throughout the cycle. It is hence critical that assessment of isoflavone influence on steroid receptor expression and distribution in intact cats be conducted in animals during the same stage of the oestrous cycle, in order to standardise the effects of endogenous oestrogens.

1.7.4.2. *Histological changes during normal oestrous cycling*

Hysterographic and histological changes during the normal oestrous cycle of the domestic cat have been well described (Chatdarong *et al.* 2005; Zambelli and Cunto 2005). Reported oestrogen-induced changes in the reproductive tract, both at the macroscopic and microscopic level, appear relatively consistent between mammalian species, especially when dose-, tissue- and time-dependent variation is accounted for. The type of change, if any, elicited following isoflavone administration may provide some indication of their mechanism of action and their potential oestrogenic or anti-oestrogenic capacity may be evaluated from histological changes associated with their administration.

1.7.4.2.a. *The Vagina*

Changes reported in the domestic cat vagina during the follicular phase (involving elevated circulating E_2 concentrations) have included a reduction of cervix height, relaxation of the cervix, increased dorsal medial folding, and elongation of the vaginal vault (fornix) (Arthur *et al.* 1989; Zambelli and Cunto 2005). During the luteal phase (when P_4 is elevated), the vaginal mucosa receives decreased blood supply, while secretions are also seen to decrease (Arthur *et al.* 1989; Feldman and Nelson 1996). The vaginal epithelium has been shown to become cornified, and mucus to be secreted in greater quantities under the influence of oestrogen, compared to during quiescent states (Arthur *et al.* 1989; Feldman and Nelson 1996).

1.7.4.2.b. *The Uterus*

Studies have reported that, whilst under the influence of E_2 during the follicular phase, the feline uterus and uterine horns becomes enlarged with a congested, hyperaemic endometrium, and has increased secretory activity in preparation for implantation (Arthur *et al.* 1989; Feldman and Nelson 1996; Chatdarong *et al.* 2005). During the oestrous cycle, the endometrium was at its thickest in the follicular phase, although no significant difference was found between luteal and follicular phase thickness (Chatdarong *et al.* 2005). Myometrial thickness was significantly greater in both the luteal and follicular phase, compared to the inactive phase (Chatdarong *et al.* 2005).

Luminal epithelial cell height was significantly greater during follicular and luteal phases, compared to the inactive phase, but glandular epithelium cell height was increased most significantly in the luteal phase (Chatdarong *et al.* 2005). Corresponding structural changes occurred in the luminal epithelium, which became pseudostratified in the follicular phase, and progressed to being hyperplastic in the luteal phase (Chatdarong *et al.* 2005). The glandular epithelium was pseudostratified in the luteal phase, with only minimal changes observed in the follicular phase (Chatdarong *et al.* 2005). The degree of glandular proliferation, secretion and dilatation was also greater in the luteal phase, and

cystic formation was slightly elevated (Arthur *et al.* 1989; Feldman and Nelson 1996; Chatdarong *et al.* 2005).

However, despite the apparent differentiation and proliferation in the reproductive tract during oestrus, cellular proliferation (as indicated by proliferative cell nuclear antigen) was unaffected by oestrous stage (Chatdarong *et al.* 2005). The high degree of variation observed in this parameter within stages of the cycle was the most likely reason for the lack of significant differences.

1.7.4.2.c. *The Oviducts and Ovaries*

Minimal follicular growth was evident during anoestrus or inter-oestrus in feline tissue, and any corpora lutea present were regressed and non-functional (Arthur *et al.* 1989; Feldman and Nelson 1996). However, during oestrus, ovaries become enlarged with raised vesicular follicles visible on their surface (Arthur *et al.* 1989; Feldman and Nelson 1996). Oviducts become hypertrophied and fully ciliated with increased secretion during the follicular phase of the cycle (West *et al.* 1977). Similar effects were observed in OVX cats treated with exogenous E_2 (West *et al.* 1977), and the mode of basal body and cilia formation in domestic cats appears to be essentially the same as in oviducts of other species (Verhage and Brenner 1975).

Corpora lutea are reported to be present on the ovarian surface following ovulation (Arthur *et al.* 1989; Feldman and Nelson 1996) and these vesicles release P_4. Endogenous and exogenous P_4 has been shown to cause deciliation, decreased cell height, and secretion, in the oviducts of cats (West *et al.* 1977). Indication of P_4 influence in the pregnant and E_2-primed OVX cat treated with exogenous P_4 was inferred by the presence of dense secretory granules, surrounded by light halos in the glandular epithelium of cat uteri (West *et al.* 1977).

1.7.4.3. *Folliculogenesis*

Folliculogenesis in the domestic cat is easily studied due to the presence of large numbers of ovarian follicles at all stages of development in the feline ovary (Bristol-Gould and Woodruff 2006). It is now known that primary oocyte formation in the cat occurs between days 40 – 50 of gestational life, and is completed approximately 8 days after birth (Bristol-Gould and Woodruff 2006). Each primordial follicle contains an oocyte arrested in the first meiotic prophase, which does not resume development until triggered by gonadotrophins (Bristol-Gould and Woodruff 2006).

The TGF-β superfamily has been shown to play an important role in balancing the growth and apoptosis involved in follicular development in the domestic cat (Bristol and Woodruff 2004). Isoflavones are reported to be capable of modulating TGFβ expression and activity (see Section 1.5.2.6.b) and this may represent a mechanism for isoflavone activity in the reproductive tract of cats.

Conversely, unlike findings in bovine pregnancy (Woclawek-Potocka *et al.* 2005ab), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was not luteolytic during the early luteal phase in the cat (Wildt *et al.* 1979). Therefore, if the ability of isoflavones to increase $PGF_{2\alpha}$ seen in cattle (Woclawek-Potocka *et al.* 2005ab) was also to occur in domestic cats, it is unlikely that this will be translated into ovarian changes.

1.7.4.4. *Steroid receptor expression and growth factors*

The expression and distribution of steroid receptors has been found to vary according to the stage of oestrous cycle in the domestic cat (West *et al.* 1977; Li *et al.* 1992). Overall ERα expression in the uterus and oviducts was reported to be low during anoestrus, increased during oestrus, and reduced during pregnancy and the luteal phase (West *et al.* 1977). Oestrus cats exhibited significant concentrations of ERα and PR in the luminal and glandular epithelium, as well as stromal fibroblasts (Li *et al.* 1992).

In addition to changes in receptor expression, distributional effects have also been observed according to stage of cycle. The luminal surface epithelium was shown to express significant concentrations of ERα or PR only during oestrus (the follicular phase) (Li *et al.* 1992). These receptors were not detected in these cell types following ovulation or exogenous P_4 administration (Li *et al.* 1992). However, in the glandular epithelium, ERα was expressed in the nuclei of all cells, as well as in the stromal fibroblast cells during both follicular and luteal phases (Li *et al.* 1992), albeit in decreased concentrations during pregnancy (West *et al.* 1977). Progesterone receptor expression disappeared from the luminal epithelial cell nuclei following ovulation, but was maintained in the stromal fibroblast cells (Li *et al.* 1992). The decreased ERα concentrations observed during pregnancy were thought to be the result of increasing P_4 concentrations, since E_2 concentrations were high enough to maintain ERα (West *et al.* 1977). These studies investigating ER expression of the domestic cat uterus were conducted prior to the discovery of ERβ (first cloned in the rat by Kuiper *et al.* 1996) and therefore no data exists to date as to the distribution and expression of this isoform.

The surface (luminal) epithelium of OVX or pregnant domestic cat uteri is not known to contain ER or PR (Li *et al.* 1992). However, this tissue still becomes convoluted in response to both endogenous and exogenous E_2 during these phases. The glandular epithelium was also responsive to E_2 and became hypertrophic and increasingly differentiated (Chatdarong *et al.* 2005). It has been suggested that the waviness and coiling of the uterine lumen during these phases was a prostagenic effect, related to the proliferation of endometrial glands (Chatdarong *et al.* 2005). The presence of ERs and PRs in glandular tissue may explain the pathway for such histological changes. However, since luminal epithelium contains significant concentrations of EGF receptor it is thought that EGF plays a more important role in modifying the luminal epithelium (which is devoid of ERα and PR), via the interaction of TGFβ and EGF with ovarian steroids (Boomsma *et al.* 1997). Receptors for EGF are also found in the glandular epithelium. These are likely to be significant factors in the steroid-induced changes observed in the

domestic cat reproductive tract during normal oestrous cycles, and following exposure to exogenous hormones (Boomsma *et al.* 1997).

Interestingly, although EGF functions under E_2-influence to control the growth and differentiation of domestic cat uteri and oviducts, neither growth factor nor the EGF receptor were affected in expression or distribution by steroid treatment in OVX cats (Boomsma *et al.* 1997). The only apparent response to steroid treatment reported in OVX cats was the appearance of dense deposits of TGFβ in the epithelium and stroma following P_4 treatment, which has also been noted in other species (Boomsma *et al.* 1997). The ability of isoflavones to modulate growth factor activity and expression in other species may therefore not elicit similar changes in the expression of these factors in the cat.

Structural and functional similarities between human and feline ERα supports the use of the cat as a model for human ERα (Cardazzo *et al.* 2005). The GenBank (2006) database on homology has reported that feline and human ERα have 95% homology, whilst the human and feline PR also share 95% homology. The homology of feline and mouse PGR was lower (89%), whilst feline and mouse ERα share 100% homology (GenBank 2006). This is important when extrapolating effects on steroid receptor expression in the cat from rodent or human studies. However, as discussed previously, genomic similarities may not necessarily manifest parallel responses at the tissue or whole-animal level.

1.8. Comparative Nutrition and Reproduction in the Domestic Cat (*Felis catus*) and Cheetah (*Acinonyx jubatus*): Factors Related to the Potential Impacts of Dietary Isoflavones

It has been established that isoflavones are absorbed by both cheetahs and domestic cats, and moreover that isoflavones have been associated with changes to hepatic and endocrinological parameters in both species. In order to determine the potential of isoflavones to influence reproductive and hepatic health in captive cheetahs controlled trials must be conducted. Since the cheetah is an endangered species (IUCN 1996) and captive numbers are therefore low, it is necessary to utilise a model species to investigate the consequences of long-term isoflavone exposure in felids. The domestic cat is frequently used a model for nutritional and reproductive studies in non-domestic felids but the question of whether it is a suitable model species for the study of isoflavone-induced effects in the reproductive and hepatic system of cheetahs remains unanswered. Phylogenetically-speaking the domestic cat is only a very distant relative of the cheetah since the cheetah was one of the first of the modern cats to diverge from their common ancestor, whilst the domestic cat was one of the last (Turner and Antón 1997).

All members of the family Felidae are specialist meat-eaters, and the differences seen in modern felids compared to other mammals are likely to be consequences of their early dietary specialisation. Their metabolic "deficiencies" may in fact represent adaptations in themselves and have been the subject of exhaustive review elsewhere (Morris 2001; Zoran 2002). Within the felid taxon, nutritional and reproductive physiology is likely to differ, although the degree of interspecific variation is not well established. It is beyond the scope of this thesis to investigate the complete array of nutritional and reproductive

specialisations of felids. As such, effort is made to discuss factors relevant to isoflavone exposure and biological activity in cats and cheetahs.

1.8.1. Anatomical Comparison of the Digestive System

The cheetah is frequently cited as being anatomically different to other cats, due to its extreme adaptation as a sprinter. The larger nasal passages, bronchi, lungs, heart and adrenals have been discussed as adaptations to short bursts of rapid speed (Eaton 1974; Marker and Dickman 2003). Yet, in contrast to this previous assumption, assessment of the cheetah anatomy did not yield a great number of differences to that of the domestic cat when organ dimensions were scaled relative to the total body weight or length. Nonetheless, some noteworthy differences were found to exist between cheetahs and other large felids, suggesting that the cheetah may be more anatomically similar to the domestic cat than it is to other large felids. This could be explained by the early divergence of the *Acinonyx* line from the ancestral cat lineage (Turner and Antón 1997; Mattern and McLennan 2000).

1.8.1.1. *The Gastrointestinal Tract*

The small intestine of the domestic cat is reported to be approximately 3.4 times greater than its body length, whilst the cheetah's was only 2.9 times greater in length (Maskell and Johnson 1993; Sturgess *et al.* 2001; Cheetah Conservation Fund pers. comm. 2006). The large intestine of the domestic cat was also elongated, compared to the cheetah (0.8 times *versus* 0.73 times the body length, respectively) (Maskell and Johnson 1993; Sturgess *et al.* 2001; Cheetah Conservation Fund, pers. comm. 2006). Although the absolute differences between the two species are not marked, the potential differences in gastrointestinal tract transit time and microflora population size warrant consideration. Enhanced absorptive, and/or bioconversion capacity could be predicted for the domestic cat as a result of its longer gastrointestinal tract.

Comparison of microflora populations in the proximal intestine of cheetahs and domestic cats has not been reported in the literature. As the proximal intestine is known to be important in the hydrolysis and absorption of dietary isoflavones the microflora content of these tissues may provide some indication of the potential for microbial degradation and/or metabolism of isoflavones consumed by felids.

However, cheetahs had similar rectal gram-negative bacterial populations to domestic cats, but a considerably lower incidence of gram-positive bacteria in rectal cultures (Howard *et al.* 1993). *Bacterioides* and *Lactobacillus* species made up 46.7% and 36.7% (respectively) of domestic cat rectal isolates, whereas these species were absent from cheetah isolates (Howard *et al.* 1993). These bacterial species have been reported to be associated with isoflavone metabolism (see Section 1.3.1.) and their absence from cheetah rectal isolates may be reflected in a lower or modulated metabolic capacity in the cheetah. However, the primary site of isoflavone metabolism in felids is unknown. As such, it is unclear whether the varying microflora in the rectum of cheetahs and cats will confer any significant differences in isoflavone metabolism.

1.8.1.2. *The liver*

Liver size (relative to body size), anatomical location, and ultrasonographic echogeneity, are equivalent in cheetahs and the domestic cat (Spector 1956; Carstens *et al.* 2006; Cheetah Conservation Fund, pers. comm. 2006). Interestingly though, the relative liver size of the cheetah was greater than compared to other large felids such as the tiger, jaguar and leopard (Spector 1956). However, anatomical similarities between the domestic cat and cheetah liver do not necessarily infer similar hepatic function or responses to exogenous compounds, particularly isoflavones.

1.8.2. Physiological Comparison of Nutritional Biochemistry

The reference range for normal values of a number of serum parameters measured in adult cheetahs and domestic cats have overlapping values. Parameters without notable differences in range values between these two species include serum calcium, blood urea nitrogen, total bilirubin and amylase. However, greater circulating concentrations of glucose, cholesterol, creatinine, ALP, AST, and ALT were reported for the cheetah, than the domestic cat (Bechert *et al.* 2002; Plumb 2002; Wack 2003). In contrast, serum concentration of GGT and lipase concentrations were typically lower in the cheetah than the domestic cat (Bechert *et al.* 2002; Wack 2003). These differences may represent variation in hepatic physiology between the two species, although the high incidence of liver disease in captive cheetahs (Munson *et al.* 2005) may have introduced bias in the sampling of hepatic enzymes. The clinical significance of these differences is, hence, difficult to interpret. It is therefore, important to use species-specific reference ranges, and/or multiple samples over time, when evaluating individual species' hepatic biochemistry.

1.8.2.1. *Metabolism of Secondary Plant Compounds*

Where studied, the shared carnivorous diet and highly conserved genome of the Felidae has resulted in analogous nutritional metabolic pathways. As a consequence of the exclusion of plant material from the diet of carnivores, the metabolic pathways typically used by omnivores to de-toxify secondary plant compounds have been shown to be deficient in felids (Court and Greenblatt 2000). These compounds can then become toxic to both domestic cats and non-domestic felids (Ashton and Jones 1979; Court and Greenblatt 2000; Section 1.7.2.1.).

Lions (*Panthera leo*), as well as other non-felid carnivores, fail to conjugate phenols to glucuronic acid (French *et al.* 1974), but the cheetah has not been studied. However, similar genetic mutations are anticipated to have occurred across the Felidae family

without evolutionary disadvantage. Indeed, mutations of the gene encoding for these metabolising enzymes were common to the domestic cat and margay (*Leopardus wiedii*) (Court and Greenblatt 2000). Likewise, *N*-acetyltransferase, a cytosolic enzyme involved in the metabolism of a number of xenobiotics, exhibited only low activity in the domestic cat (Trepanier *et al.* 1998). The unusual presence of only a single gene encoding for this protein is common to all felids studied, including both the domestic cat and cheetah (Trepanier *et al.* 1998). It appears that the cheetah and domestic cat may share a common deficiency in glucuronidation capacity and most likely have comparable metabolic pathways for the handling of xenobiotics and phenols. This suggests that the domestic cat may provide a suitable model species for the prediction of isoflavone pharmacokinetics and bioavailability in the cheetah.

1.8.3. Comparison of Relevant Reproductive Parameters

1.8.3.1. *Fertility and fecundity*

The domestic cat is generally considered to be a fertile model species, with a high capacity for reproduction (Griffin 2001; Pelican *et al.* 2006). However, intensely-managed colonies, such as laboratory-maintained research colonies, are prone to inbreeding and resultant genetic diseases which may compromise their fertility (Pelican *et al.* 2006). Interestingly, these inbred cats may offer a more suitable animal model for the investigation of disease and reproductive failure in genetically impoverished, endangered felid species, such as the cheetah (Pelican *et al.* 2006).

1.8.3.2. *Reproductive cycles*

The cheetah and domestic cat are polyoestrous (Arthur *et al.* 1986; Brown *et al.* 1996; Feldman and Nelson 1996; Brown 2006), whereby oestrous cycling will continue until the female becomes pregnant, undergoes a pseudo-pregnancy, or environmental cues instigate the cessation of cycling (see Figure 1.6). The oestrous cycle of the cheetah

spans 10 – 14 days on average (Fowler 1986; Asa *et al.* 1992; Laurenson *et al.* 1992; Brown *et al.* 1996), which is markedly shorter then seen in other large felid species, but similar to that of the domestic cat (Asa *et al.* 1992; Brown *et al.* 1996; Brown 2006). Within normal cycles of the cheetah and domestic cat, E_2 was shown to increase for approximately 4 days (range 1 – 18) (Brown *et al.* 1996; Wielebnowski *et al.* 2002). However, cheetah cyclicity is known to be highly variable (ranging from 3 – 27 days in length), with a high incidence of prolonged anoestrus (lasting up to 170 days) in captivity (Asa *et al.* 1992; Brown *et al.* 1996; Wielebnowski *et al.* 2002). This high variability in cycle duration may potentially relate to the induced-ovulatory nature of these felids, since oestrous cyclicity in the majority of Felidae appears less well regulated than in spontaneous ovulators (Brown *et al.* 1996). The increased level of natural variability in felid cyclicity indicates a potentially reduced opportunity for the detection of isoflavone-induced cycle irregularities, compared to other mammals.

1.8.3.3. *Puberty onset and sexual maturity*

Cheetahs typically undergo puberty between 14 – 16 months of age (Fowler 1986), whereas the domestic cat experiences puberty at 6 – 9 months (Arthur *et al.* 1989; Feldman and Nelson 1996). Relative to longevity, cheetahs are calculated to spend between 9.72 – 12.12% of their lives in a pre-pubescent state, whilst domestic cats spend only 2.94 – 5.77% of their lives in this state. Perturbation of puberty onset by dietary isoflavones may therefore have a greater impact on life-time reproductive capacity in cheetahs than the domestic cat.

1.8.3.4. *Ovulation induction*

Cheetahs and domestic cats are both described as induced ovulators (Banks and Stabenfeldt 1982; Concannon *et al.* 1989; Laurenson *et al.* 1992; Brown *et al.* 1996), meaning that vaginal stimulation is necessary to elicit a significant surge in luteinising hormone concentrations, required to bring about ovulation (Banks and Stabenfeld 1982;

Concannon *et al.* 1989; Brown 2006). Ovulation is indicated by a rise in P_4 in both domestic cats and cheetahs (see Figures 1.6 and 1.7), and occurs in an all-or-nothing fashion, with over 80% of mature follicles rupturing (Banks and Stabenfeldt 1982; Concannon *et al.* 1989).

However, spontaneous ovulation has occasionally been reported in the cheetah (Asa *et al.* 1992; Brown *et al.* 1996; Howard *et al.* 1997; Wielebnowski and Brown 1998; Laurenson *et al.* 1992), and domestic cat (Gudermuth *et al.* 1997). Such events may occur in response to environmental triggers (psychosocial), or non-sexual physical stimulation (Brown *et al.* 1996). It could be predicted that ovulation in the cheetah and domestic cat is under similar control and therefore likely to exhibit similar, if any, responses to the administration of exogenous oestrogen such as isoflavones.

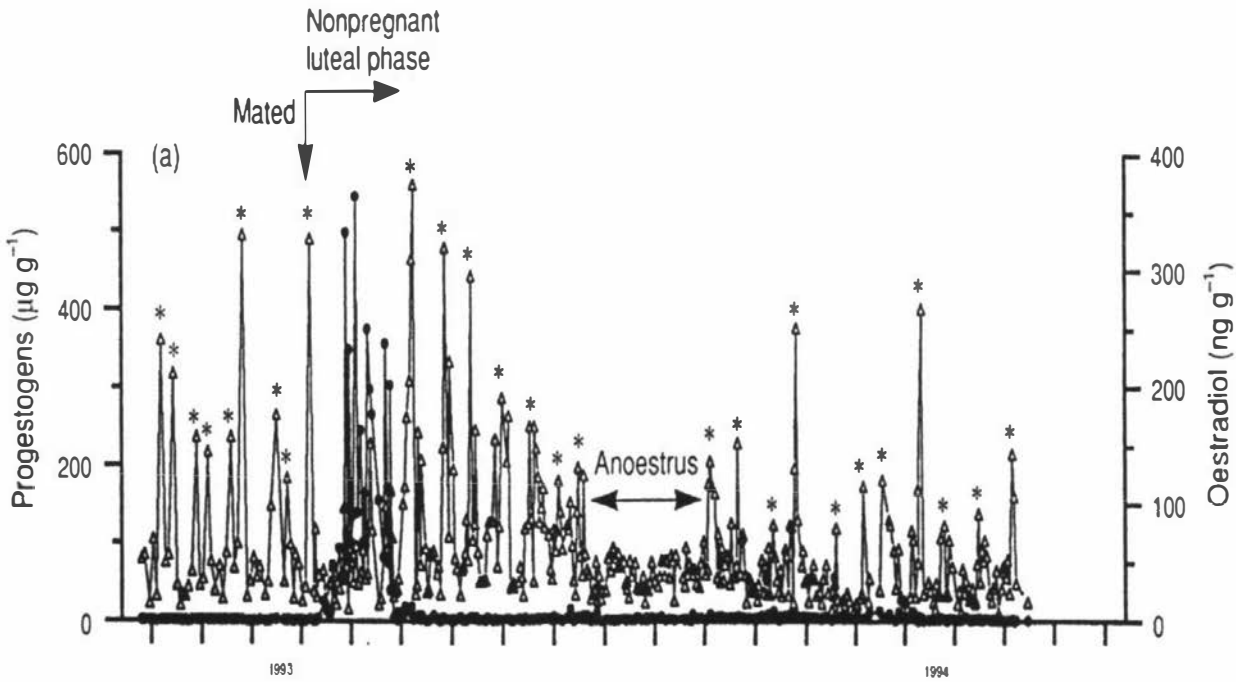


Figure 1.6. Faecal oestradiol (Δ) and progesterone (●) excretion in a female cheetah after a non-fertile mating. * indicates oestrus (Brown *et al.* 2001).

Faecal P_4 concentrations have been reported to remain elevated for 38 – 41 days following spontaneous ovulation in the cheetah (Wielebnowski *et al.* 2002), whilst pseudopregnant-related P_4 elevations in the cat are typically shown to last approximately 28 - 40 days (Feldman and Nelson 1996) (see Figure 1.7). It is interesting to note that the absolute duration of pseudo-pregnancy is the same for cheetahs and domestic cats which may indicate that the functional lifespan of the corpora lutea is similar in both species.

The cheetah has an exceptional lack of exaggerated response to exogenous gonadotrophins, compared to other felids, including the domestic cat (Brown 2006). Exogenous gonadotrophins are routinely used to induce oestrus and ovulation prior to artificial insemination procedures, and typically elicit hyper-stimulated ovarian responses, with circulating oestrogen concentrations increasing several-fold over those observed during natural oestrus (Brown 2006). However, in the cheetah, oestrogen concentrations following exogenous gonadotrophin administration did not differ from those seen during natural oestrus (Brown 2006). The frequently quiescent state of cheetah ovaries may be responsible for the lack of response, since less endogenous steroids would be in circulation at the time of gonadotrophin exposure (Brown 2006). In this regards, it is possible that stimulation by environmental oestrogens could exhibit a dampened oestrogenic response in the cheetah, compared to the domestic cat.

1.8.3.5. *Steroid hormone changes of the oestrous cycle*

Changes in circulating E_2, P_4 and LH during the reproductive cycle of the domestic cat is depicted in Figure 1.8. Cheetahs and cats have been shown to be similar in their excretion of sex steroid hormones (see Figure 1.8) (Brown *et al.* 2001 and 2006), and oestrus behaviour and mating were correlated with peaks in faecal E_2 excretion in both species (Brown *et al.* 1996; Wielebnowski and Brown 1998).

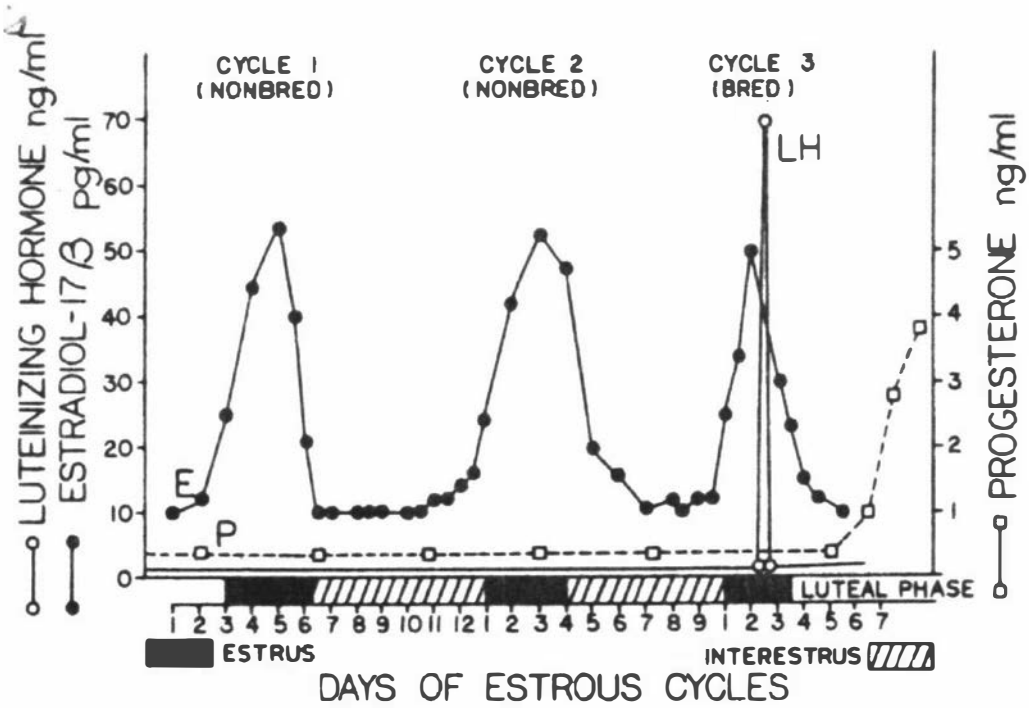


Figure 1.7. Circulating oestradiol (●), luteinising hormone (○) and progesterone (□) profile of the domestic cat oestrous cycle and ovulation inducing luteinising hormone surge following mating (Banks and Stabenfeldt 1982).

Faecal excretion studies have also determined that metabolism of E_2 was comparable between the domestic cat and cheetah (Brown *et al.* 1994). This is encouraging when attempting to use the domestic cat as a model species for the cheetah in investigations of reproductive responses to isoflavones.

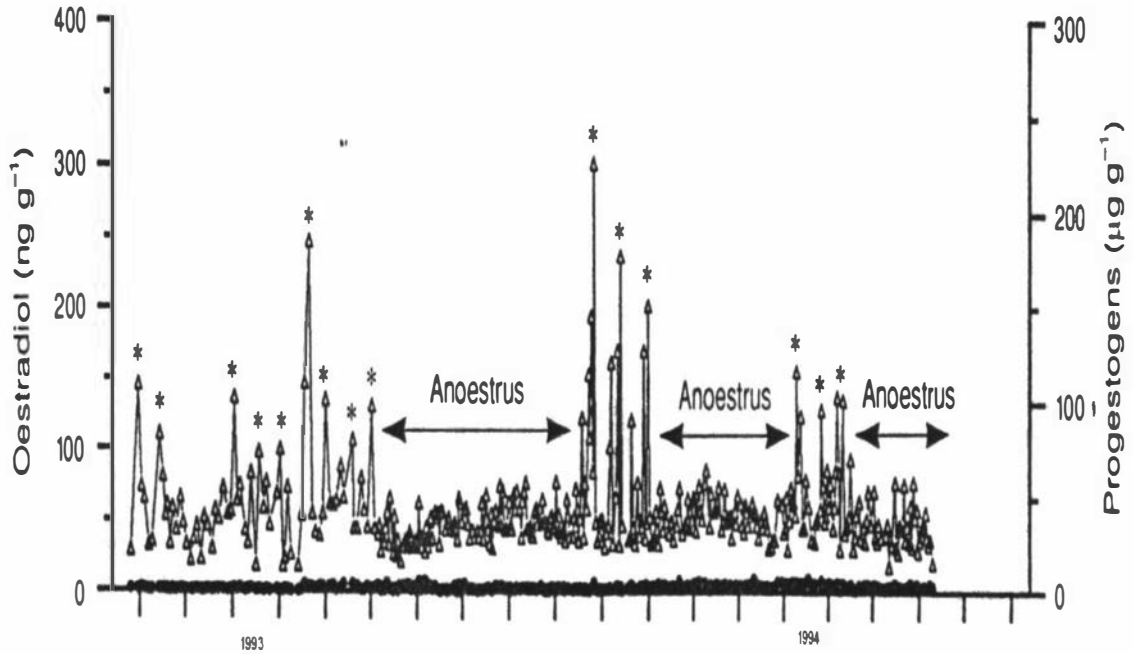


Figure 1.8. Faecal oestradiol (Δ) and progesterone (•) excretion in a female cheetah. * indicates oestrus (Brown *et al.* 2001).

1.8.3.6. *Vaginal Cytology*

Distinct cytological changes have been demonstrated to occur during the oestrous cycle of the cat and cheetah (see Figure 1.9) (Mills *et al.* 1979; Mowrer *et al.* 1975; Shille *et al.* 1979; Asa *et al.* 1992). In general, a rise in E_2 was paralleled by increasing proportions of anuclear superficial epithelial cells, followed by a leukocyte infusion, which marked the end of the follicular phase in both species (Shille *et al.* 1979; Asa *et al.* 1992). Correlation between serum E_2 and anuclear cell presence was high, although no correlation was found between serum P_4 and any vaginal cell type (Asa *et al.* 1992). The leukocyte infusion of the luteal phase in cheetahs has occasionally (Shille *et al.* 1979), but not consistently, been reported for the domestic cat (Mowrer *et al.* 1975). However, this infusion is unreported in the lion, puma or tiger, suggesting that domestic cats and cheetahs may be more comparable in this respect (Asa *et al.* 1992).

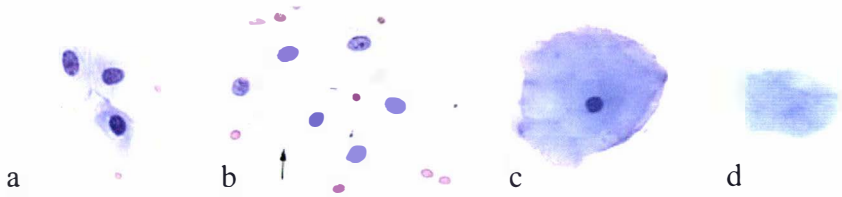


Figure 1.9. Various cellular stages of vaginal epithelium during the oestrous cycle of the domestic cat (a), parabasal cells predominate during inter-oestrus, (b), intermediate cells begin to appear in pro-oestrus and (c), nucleated superficial cells become more prominent during oestrus. Anucleated superficial cells (d), are indicative of true oestrus.

The resumption of anoestrus or inter-oestrus is reported to be indicated by a return of parabasal cells to approximately 10 - 50% of the total, a decrease in anuclear superficial cells to <10% of the total, and an increase to 40 – 50% in intermediate and nucleated superficial cells (Mowrer *et al.* 1975; Mills *et al.* 1979; Shille *et al.* 1979; Feldman and Nelson 1996). Background debris was also apparent during this stage (Mowrer *et al.* 1975; Mills *et al.* 1979; Feldman and Nelson 1996). The similarity in vaginal cytological response to endogenous oestrogen in the domestic cat and cheetah indicates that cytological responses to exogenous oestrogens are likely to be similar in these two species.

1.8.3.7. *Behaviour*

Behaviours associated with oestrus in felids are fairly typical across the taxon and are known to include rolling, rubbing, vocalizing, pacing, locomotor activity, grooming, urine spraying (both males and females), investigative activities and lordosis (Arthur *et al.* 1989; Asa *et al.* 1992; Feldman and Nelson 1996; Wielebnowski and Brown 1998). These behaviours have been correlated to elevated plasma E_2 concentrations and were indicative of changes in vaginal cytology in the domestic cat and cheetah (Shille *et al.* 1979; Asa *et al.* 1992; Wielebnowski and Brown 1998). This finding further supports the use of the domestic cat as a model animal for the cheetah.

1.9. Research Objectives

The overall goal of this thesis is to determine the potential of dietary isoflavones to influence the reproductive and hepatic system of the domestic cat, as a model for the cheetah. Within this goal, there is the opportunity to investigate the validity of the domestic cat as a model for the cheetah, in regards to its absorption and metabolism of dietary isoflavones.

1.9.1. Dietary Intake of Isoflavones by Cheetahs and Domestic Cats

The preliminary objective of this thesis is to determine the dietary intake, and predicted exposure of domestic cats in New Zealand, and captive cheetahs in international facilities, to isoflavones. Although previous studies have reported the isoflavone content of feline diets in North America, no investigation has been conducted on the isoflavone content of captive cheetah diets.

1.9.2. Isoflavone Absorption, Metabolism and Excretion in Cheetahs and Domestic Cats

This thesis will investigate the absorption, metabolism and excretion of genistein and daidzein in the domestic cat and cheetah. The pharmacokinetics of genistein has been investigated previously in domestic cats, utilising a dose exceeding that attainable from a commercially prepared diet, whilst no data exists as to the absorption or excretion of daidzein in the cat. Likewise, the absorption and excretion of either of these compounds by the cheetah has not previously been reported. Furthermore, the metabolites produced by cats and cheetahs following oral exposure to genistein and daidzein have not been previously reported.

1.9.3. Comparison of the Absorption, Metabolism and Excretion of Isoflavones by Domestic Cats and Cheetahs

In order to validate the use of the domestic cat as a model species for the cheetah, this thesis will determine the interspecific differences in isoflavone handling. This type of investigation has not been reported previously.

1.9.4. The Effects of Isoflavones in the Domestic Cat Reproductive System

No study has been conducted to determine the influence of isoflavones in the reproductive system of the domestic cat. This thesis will examine reproductive tract histology, cycle characteristics and sex steroid receptor expression in domestic cats reared on an isoflavone-containing diet from weaning until sexual maturity, compared to control cats.

1.9.5. The Effects of Isoflavones on the Domestic Cat Hepatic System

No study has been conducted to determine the influence of isoflavones in the hepatic system of the domestic cat. This thesis will investigate the ability of dietary isoflavones to alter hepatic serum chemistry and/or histology.

CHAPTER TWO:

The Dietary Isoflavone Content of Diets fed to Domestic Cats (*Felis catus*) and Captive Cheetahs (*Acinonyx jubatus*)

Section 2.1 has been published as a scientific article (Appendix 1 & 2): **Bell KM,** Rutherfurd SM, Hendriks WH. (2006). The dietary isoflavone content of commercially available domestic cat diets in New Zealand. *New Zealand Veterinary Journal* 54(3): 103 – 108. Corrigendum: *New Zealand Veterinary Journal* 54(6): 336.

2.0. Introduction

Consumption of dietary isoflavones has been tentatively linked to physiological perturbations, including reproductive impairment, modulation of hepatic function and altered thyroid hormone production in domestic cats and captive cheetahs (Setchell *et al.* 1987ab; White *et al.* 2004). Before prediction of the physiological impacts of dietary isoflavones in felids can be made, it is necessary to determine the range of isoflavone contents in diets consumed by domestic cats and cheetahs. No study exists to determine the isoflavone content of commercially prepared diets consumed by domestic cats in New Zealand, or diets consumed by captive cheetahs in international facilities. This study aimed to identify and quantify the concentration of dietary isoflavones in commercially-available diets intended for consumption by domestic cats and captive cheetahs.

2.1. Experiment One: Domestic Cat Diet Analyses

2.1.1. Aim

The aim of this study was to identify and quantify concentrations of the isoflavones genistein, daidzein, biochanin A and formononetin, in commercially-prepared domestic cat diets, sold in New Zealand.

2.1.2. Materials and methods

2.1.2.1. *Chemicals*

Solvents and acids were obtained from BDH (Poole, UK). Pure standards of genistein, daidzein, glycitein, biochanin A and flavone were obtained from Sigma-Aldrich (St. Louis, MO, USA), and formononetin from Merck BioSciences (Darmstadt, Germany). A solution containing equal and known amounts of each of the isoflavone compounds was prepared and used as a reference standard (20.8 μg/ml of each isoflavone in 10% hydrochloric acid-methanol). The reference standard (combination of all 4 standards) was stored at –4°C.

2.1.2.2. *Diet samples*

Domestic cat diets (n = 138) were obtained from supermarkets (n = 117), veterinary clinics (n = 15) and pet-food retail stores (n = 6) in New Zealand. The diets were classified into five categories, according to the following criteria: the presence/absence of soy material as described on the manufacturer's label, the presence/absence of non-soy plant material as described on the manufacturer's label, and dry matter (DM) content. Hence the diets were classified as one of either: moist with-soy, moist without-soy, dry

with-soy, dry without-soy or moist meat-only. All diet samples were freeze-dried for 72 h before being finely ground in a coffee grinder (Coffee N' Spice Grinder, Model CG-2; Breville, Oldham, UK). Each sample was mixed thoroughly and stored in a plastic bag at –20°C prior to analysis. Dry matter was determined following oven-drying samples at 105°C for 12 h. Preparation of samples, acid hydrolysis and extraction methodologies were adapted from those described by Court and Freeman (2002). All diets were analysed in triplicate.

Two grams of each sample was weighed into a 30 ml polypropylene screw-cap tube. Preliminary experiments involving a series of incubation periods, temperatures and extracting solution strength determined that a 4 h incubation period in a boiling water bath produced optimal extraction of isoflavones. Extracting solution (10 ml of 6 M hydrochloric acid:methanol at 1:9) was added to each tube, which was then capped, mixed by vortexing and incubated in a boiling water bath for 4 h. The tubes were shaken and caps tightened on a half-hourly basis to ensure adequate mixing and prevent evaporative losses of extracting solution. After incubation, the samples were quantitatively transferred to 25 ml volumetric flasks and made up to volume with extracting solution. A 2 ml aliquot of each sample was centrifuged for 10 min at 13,400 rpm in a microfuge (Minispin; Brinkmann-Eppendorf, CT, USA) and 25 µl of supernatant subsequently removed and injected onto the High Performance Liquid Chromatography (HPLC) column.

2.1.2.3. *High performance liquid chromatography (HPLC) analysis*

Analysis was conducted using a Waters Alliance (Millipore Corp, Milford MA, USA) HPLC, using a Luna 5 µ C18 reverse phase column (4.6 x 150 mm; Phenomenex, Torrance CA, USA), with an in-line 4 x 3 mm C18 guard column (Phenomenex, Torrance CA, USA). Isoflavones were detected by absorbance at 260 nm, using a Waters W486 ultraviolet (UV) detector (Millipore Corp, Milford MA, USA). Samples were injected in 95% Buffer A (10% HPLC-grade acetic acid) and 5% Buffer B (HPLC-grade

acetonitrile) and eluted using a linear gradient of 5 – 70% Buffer B over 40 min followed by a 15 min equilibration with 70% Buffer B before returning to starting conditions. Baseline resolution was achieved for all the components of the isoflavone standard. The rate of flow was set at 0.5 ml/min, and isoflavones were quantified using the Millennium software package (Waters Inc., Millipore Corp, Milford MA, USA), based on the area under the curve for each peak.

Experiments to validate the assay were conducted on three pet food samples, selected on the basis of inclusion of soy products in their lists of ingredients and demonstration of isoflavone content during preliminary experiments, as well as a soy protein isolate (Protein Technologies, MO, USA). Preliminary spiking experiments were conducted to determine the recovery of each isoflavone from a pet food matrix. Mean recoveries of each spiked compound in preliminary experiments were as follows: daidzein 95% (± 0.03), genistein 85% (± 0.01), biochanin A 87% (± 0.00), and formononetin 93% (± 0.02). Flavone spiking experiments were performed after every tenth sample during analyses, in order to estimate recovery of isoflavones for each batch of samples run concurrently. Flavone was used as an internal standard in all analyses and reported concentrations of each isoflavone in diets were adjusted for internal standard recovery. Additionally, triplicate samples of soy protein isolate were analysed within each batch of pet food samples.

2.1.2.4. *Liquid chromatography-mass spectrometry (LC-MS)*

Liquid chromatography-mass spectrometry was performed on selected fractions collected from the HPLC in order to identify peaks. The method used was as described by Wu *et al.* (2003); the only modification being that acetic acid was used in the separation mobile phase system rather than formic acid. Separation by HPLC was performed as described above (Section 2.1.2.3.) and the fraction corresponding to the peak in chromatogram thought to be glycitein was collected. Three collections were made from three separate pet foods (chosen on the basis of the clearly distinguishable peak thought to be glycitein).

Samples were then dissolved in 20% acetonitrile and 0.1% acetic acid, and injected onto a Luna C18 (150 x 2.0 mm) column (Phenomenex, Torrance CA, USA). Starting conditions were 80% Buffer A (0.1% acetic acid in water) and 20% Buffer B (0.1% acetic acid in acetonitrile). Samples were eluted over a gradient from 20 – 40% Buffer B in 40 min, followed by a further 5 min at 95% Buffer B. Ultra violet spectral data were collected using a photo-diode array, scanning at 220 – 400 nm.

Mass spectrometry was performed on an LTQ linear ion-trap mass spectrometer (Thermo, San Jose CA, USA), using positive electrospray ionisation, a needle voltage of 5.0 kV, and a capillary temperature of 275°C. The nebulising gas was nitrogen and the collision gas was ultra-pure (99.99%) helium. The mass range scanned was 150 – 1000 m/*z*. The mass spectrometer parameters were optimised using biochanin A standard (10 μg/ml), for maximum sensitivity.

2.1.2.5. *Statistical analysis*

Dry diets were defined as those containing >90% DM and moist diets defined as those containing <25% DM. No diets of intermediate DM content were analysed. Residual data was tested for normality with the Anderson-Darling test and found not to be normally distributed. Diets found to contain any isoflavone above the limit of detection of the assay were termed 'positive' for either genistein or daidzein.

Data were analysed according to the content of genistein, daidzein and total isoflavone, as well as retail price of each diet (NZ$/kg DM). Data are expressed either on a DM or metabolisable energy (ME, kcal) basis. The ME of each diet was calculated using the modified Atwater factors (crude protein x 3.5, crude fat x 8.5, and carbohydrate x 3.5), where the proportions of protein, fat and carbohydrate in each diet were recorded according to the manufacturer's labelling. Where the content of carbohydrate was not reported, it was estimated by subtraction (AAFCO 2004).

All statistical analyses were performed with SAS (version 8, SAS Institute Inc, Cary NC, USA). Univariate procedures were used to describe the median and quartiles of the data, since the data were not normally distributed and calculation of the mean was therefore inappropriate (Glantz 2005). Median and quartile data included diets with a zero value, unless otherwise specified. Proportions of isoflavone-positive diets in each category were calculated and compared for equality. Non-parametric analyses of variance were performed on isoflavone-containing diets within each group using the Kruskal-Wallis test statistic (H; $p<0.05$).

Daily intake of isoflavone was estimated according to the total content of isoflavone of each diet per unit of ME (kcal). According to the National Research Council (NRC 1986), active domestic cats have an approximate daily ME requirement of 80 kcal/kg BW. Based on this assumption, the total isoflavone content found in 80 kcal of each diet is equivalent to the total content of isoflavone consumed by a domestic cat per kg BW.

2.1.3. Results

An HPLC-based assay was developed to identify and quantify the concentrations of the isoflavones genistein, daidzein, biochanin A and formononetin in domestic cat diets. Absorbance was linear over the range of 0.125 μg/ml to 625 μg/ml for all isoflavones tested (R^2 ranged from 0.943 for biochanin A to 0.998 for genistein). Glycitein could not be adequately separated and could not, therefore, be quantified. Mass spectrometry suggested that glycitein, if present, occurred only in trace amounts.

The limit of detection of the assay was 1.56 μg/g, as defined by evaluation of the linearity of standard curves following serial dilutions of isoflavone standards and the peak signal-to-noise ratio in preliminary trials. Samples with concentrations of isoflavone below the limit of detection were assigned a value of zero. For the purposes of comparison of diet

isoflavone content between diet categories, only isoflavone-positive diets, i.e. those diets containing isoflavones above the limit of detection, were included. The average intra-assay coefficient of variation (CV) was 10% for daidzein, 7% for genistein, 9% for biochanin A and 11% for formononetin (n = 3). The inter-batch CV, determined by repeated analysis of triplicate samples of the soy protein isolate at regular intervals throughout the period of analyses of the diets, was 7%. The mean (± standard error of the mean, SEM) content of isoflavone of the soy protein isolate was 700 (± 26.3) mg/kg DM, which is within the reported range of 373 – 1875 mg/kg DM for soy protein isolate (Mazur 1998).

No category of diet was devoid of isoflavones. Isoflavones were detected at quantifiable concentrations in 104/138 (75%) of all diets tested. More dry diets (127/138; 92%) contained isoflavones at quantifiable concentrations compared with moist diets (83/138; 60%, $p < 0.01$). Overall, the most common isoflavone was daidzein, which was quantifiable in 97/138 (70%) of all diets, followed by genistein (66/138; 48%), biochanin A (25/138; 18%) and formononetin (7/138; 5%).

When comparing diet categories, the moist with-soy category was found to have the greatest proportion of isoflavone-containing diets (7/7; 100%). Significant differences were detected between isoflavone occurrences within diet categories. The lowest proportion of isoflavone-positive diets was found in the moist meat-only category (see Table 2.1).

No significant differences were found between dry diets with- and without-soy as an ingredient on the label. When categories of diet were assessed according to the proportion of genistein- and daidzein-positive diets in them, the same pattern of greatest to lowest incidence of isoflavones and significant differences between categories was observed, with the exception that no significant difference was evident between daidzein-positive proportions in the dry without-soy and moist with-soy categories (Table 2.1).

Of the isoflavone-positive diets, 33/104 (32%) contained daidzein and no detectable genistein. Of these, 30/33 (91%) were devoid of soy in their ingredient list and the remainder were in the dry with-soy category. Conversely, only 2/104 (2%) of isoflavone-positive diets contained genistein but no detectable daidzein, one of which contained soy in its ingredient list and one of which did not. Only 1/104 (1%) of isoflavone-positive diets contained formononetin without its precursor, daidzein.

Category of diet[a]	n	Genistein-positive diets (%)	Daidzein-positive diets (%)
Moist meat-only	23	17^{x}	39^{x}
Moist without-soy	43	26^{x}	53^{x}
Dry without-soy	52	63^{z}	90^{y}
Moist with- soy	7	100^{y}	100^{y}
Dry with-soy	13	85^{yz}	85^{y}

Table 2.1. Percentage of domestic cat diets within defined categories[a] that contained detectable amounts of genistein and daidzein. Values with different super-scripts, within columns, were significantly different ($p < 0.05$)

A greater proportion of diets (8/104; 8%) contained biochanin A without its precursor, genistein, and biochanin A was found in 3/104 (3%) diets containing genistein but not daidzein.

2.1.3.1. *Isoflavone content of domestic cat diets*

The diet categories moist meat-only, moist without-soy, and moist with-soy, exhibited right-skewed distributions (skewness statistics 1.10, 2.21, and 1.86, respectively and kurtosis test statistics of 0.95, 3.48, and 2.92, respectively). Diets in the dry without-soy and dry with-soy categories displayed left-skewed distributions (skewness statistics 0.25 and 0.71, respectively, and kurtosis statistics -1.51 and -1.02, respectively). Of the diets found to contain quantifiable concentrations of at least one of the four isoflavones

assessed in this study, the median total content of isoflavones was greatest in the moist with-soy category (71.1 mg/kg DM) and lowest in the moist meat-only category (3.24 mg/kg DM) ($p < 0.01$). All other categories of diet had significantly greater median total isoflavone contents than the moist meat-only category. No significant differences were evident between the four categories of diet that contained some form of plant material.

When all diets were assessed together, regardless of diet category, but including only isoflavone-positive diets, the median total content was 8.86 mg/kg DM (range 1.65 – 400.28 mg/kg DM). When the total isoflavone content was assessed on an ME basis, a similar pattern of distribution was evident, although statistical analysis was not performed on data in this form (Table 2.2; includes diets without detectable isoflavones).

	Total isoflavone content (mg/kcal ME)				Predicted isoflavone intake of domestic cats (mg/kg BW)			
Category of diet	Minimum	Median	Upper quartile (75%)	Maximum	Minimum	Median	Upper quartile (75%)	Maximum
Moist meat-only	0	0.0004	0.0007	0.002	0	0.03	0.06	0.17
Moist without-soy	0	0.0007	0.002	0.07	0	0.05	0.12	5.48
Moist with-soy	0.01	0.02	0.02	0.03	1.04	1.47	1.96	2.27
Dry without-soy	0	0.002	0.03	0.10	0	0.18	2.38	8.13
Dry with-soy	0	0.004	0.04	0.06	0	0.29	3.17	4.66

Table 2.2. Total isoflavone content of domestic cat diets and predicted domestic cat isoflavone intake, according to diet category. Medians and quartiles reported as data were not normally distributed.

The median content of genistein was highest in moist with-soy diets (39.4 mg/kg DM), which was greater than in moist meat-only (0 mg/kg DM; $p < 0.01$) and dry without-soy

(3.08 mg/kg DM; $p < 0.05$) diets. The median content of genistein in dry with-soy diets (23.4 mg/kg DM) was greater than in moist meat-only diets (0 mg/kg DM; $p < 0.01$), but not significantly different from that of any other category. Dry diets without soy had a higher median content of genistein (3.08 mg/kg DM) than moist meat-only diets (0 mg/kg DM; $p < 0.01$). Median total isoflavone content was greater in the diets that contained soy (67.4 mg/kg DM) than in those that did not (4.15 mg/kg DM; $p < 0.01$).

The mean ratio of genistein: daidzein was 1.37 (± 0.13). Figure 2.1 illustrates the distribution of the total content of isoflavone within categories of diet (including diets with no detectable isoflavones).

The median content of daidzein was also highest in moist with-soy diets (30.7 mg/kg DM), and greater in those than in moist meat-only diets (2.51 mg/kg DM; $p < 0.01$), which had the lowest median daidzein content; the median content of daidzein in moist with-soy diets was not significantly different from that of any other category. Dry with-soy diets contained 18.9 mg/kg DM daidzein, more than moist meat-only diets (2.51 mg/kg DM; $p < 0.01$). Dry without-soy diets contained more daidzein (7.60 mg/kg DM) than moist meat-only diets (2.51 mg/kg DM; $p < 0.01$) and moist without-soy diets (3.06 mg/kg DM; $p < 0.05$).

Biochanin A was present in diets from all categories except moist with-soy diets. Formononetin was present in diets from all categories except moist with-soy and moist meat-only diets. Neither of these two isoflavones was found in concentrations exceeding 20 mg/kg DM. The median content of biochanin A was higher in dry with-soy diets (1.39 mg/kg DM) than in moist with-soy diets (0 mg/kg DM; $p < 0.05$).

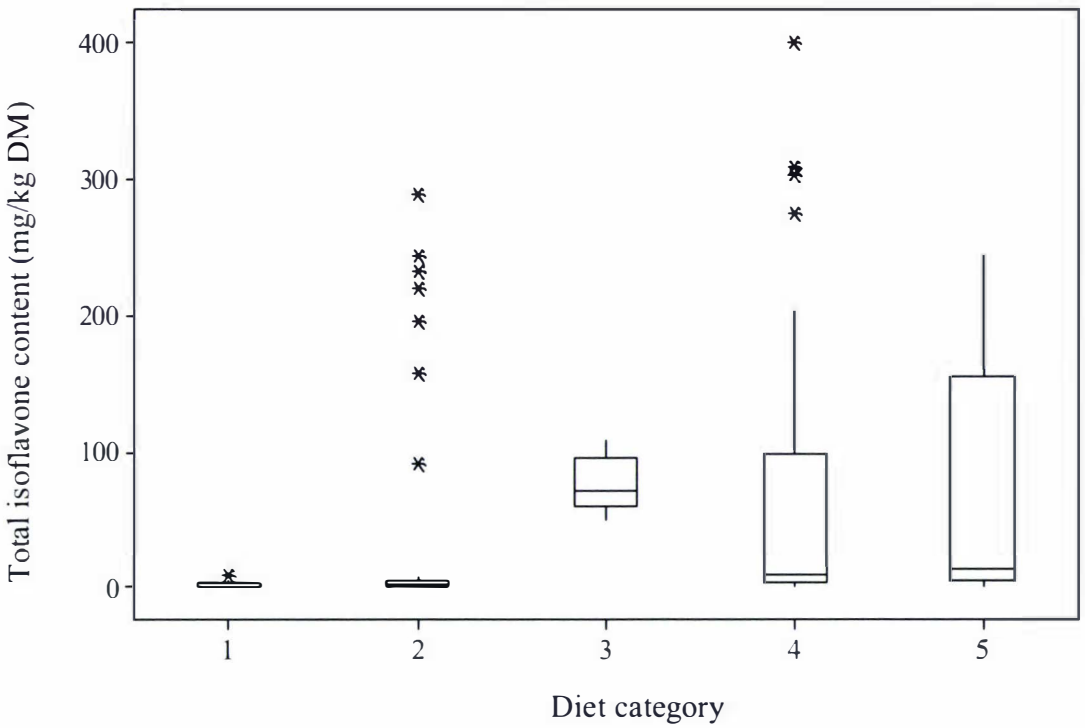


Figure 2.1. Box plot of the total isoflavone content (mg/kg DM) of domestic cat diets, according to diet category. The outliers are indicated by * whilst the upper whisker extends to the highest data value within the upper limit (upper quartile + 1.5). The top of the box represents the third quartile (75% quartile), and the bottom of the box represents the lower quartile (25% quartile). The median is represented by the line within the box. The lower whisker extends to the lowest value within the lower limit (lower quartile + 1.5).

2.1.3.2. *Average intake of isoflavone by domestic cats*

Overall, the median calculated intake by domestic cats was 0.11 mg/kg body weight (BW) (including all 138 diets). The median calculated intake of isoflavone by cats was greatest for moist with-soy diets, followed by dry with-soy diets, dry without-soy, moist without-soy, and lowest in moist meat-only diets (Table 2.2). Due to the skewed nature of the distribution of isoflavones within categories of diet, the upper percentile (75%) did not follow the same pattern. Upper percentile intakes were greatest for dry with-soy, followed by dry without-soy, moist with-soy, moist without-soy, and lowest in moist meat-only diets.

Of all the diets tested, 10/138 (7%) would provide domestic cats with an estimated total daily intake of isoflavones of 1.00 – 1.99 mg/kg BW, and 26/138 (19%) with an intake of > 2.00 mg/kg BW. Of the dry diets, 19/65 (29%) would provide cats with daily intakes > 2 mg/kg BW, whereas 11/113 (10%) of the moist diets would provide intakes of > 2 mg/kg BW. The maximum estimated daily intake of isoflavones was 8.13 mg/kg BW, for a dry without-soy diet (Table 2.2).

2.1.3.3. *Price and content of isoflavones in domestic cat diets*

Among the moist diets, those that included soy in their list of ingredients were more expensive than those without soy ($p < 0.01$). However, the cost of dry diets with soy was not significantly different from those without soy. When diets were ranked according to their price, a significant difference in total content of isoflavone was evident ($H = 72.7$; $p < 0.01$). The median concentration of isoflavones was higher in the cheapest diets (NZ$2.0–10.0/kg DM).

When assessed by category of diet, moist with-soy diets had the highest median price (NZ$41.8/kg DM), whilst moist without-soy diets had the lowest median price (NZ$11.5/kg DM). The median price of moist meat-only diets was NZ$17.3/kg DM, while that of dry without-soy diets was NZ$14.4/kg DM, and that of dry with-soy diets was NZ$12.0/kg DM.

2.1.4. Discussion

Isoflavones were found to be constituents of the majority of domestic cat diets sampled from supermarkets, veterinary clinics and pet-food retail stores in New Zealand. However, the concentration of isoflavones detected varied significantly between diet categories. Over 75% of the diets evaluated contained at least one of the four isoflavones

investigated, which is considerably higher than the 46% of domestic cat diets (Court and Freeman 2002) or the 57% of canine diets (Cerundolo *et al.* 2004) reported for North America.

The limits of detection of the assays used were similar between previous studies and the present study, and as only 9/138 diets evaluated in this study contained quantifiable concentrations of biochanin A or formononetin without genistein or daidzein, the exclusion of those isoflavones in analyses by Court and Freeman (2002) cannot explain the higher incidence of isoflavones found in domestic cat diets in New Zealand. Although the sample size was greater in this study than in the studies conducted in North America, it is reasonable to conclude that domestic cat diets in New Zealand are more likely to contain isoflavones than those in North America.

A greater proportion of dry domestic cat diets contained isoflavones than moist domestic cat diets, which may be due to more widespread use of plant material in the dry diets. Plant material is used to extend the shelf life of dry diets and provides a necessary source of carbohydrate for the expansion and cooking processes of dry diets (Zoran 2002). Previous evaluations of the isoflavone content of companion animal diets have found a significant correlation between soy and isoflavone content (Court and Freeman 2002; Cerundolo *et al.* 2004). Findings of this study also demonstrated a relationship between the total content of isoflavone and the inclusion of soy in domestic cat diets.

However, dry domestic cat diets where soy was not listed as an ingredient on the label but which contained non-soy plant material, had isoflavone contents similar to dry diets which listed soy as an ingredient. Additionally, domestic cat diets which did not list any plant material, soy or otherwise, in the list of ingredients were not necessarily devoid of isoflavones. These findings may be explained by differences in statutory labelling requirements between North America and New Zealand, the latter not requiring the specification of soy products on the manufacturer's label. Hence, a proportion of the

domestic cat diets in the category 'without-soy' may actually contain soy. Consequently, the presence or absence of soy on a list of ingredients does not appear to be a reliable indicator of the content of isoflavones in domestic cat diets available in New Zealand. A similar finding was reported by White *et al.* (2004).

Daidzein was the most frequently detected isoflavone, followed closely by genistein, in domestic cat diets. Biochanin A and formononetin contributed only minor proportions towards the total isoflavone content of all diets assessed. Interestingly, a significant proportion, *viz* 32% (33/104), of all isoflavone positive domestic cat diets was found to contain daidzein but not genistein. This is type of isoflavone profile is not typically reported for soy (Setchell *et al.* 2001) and may indicate a processing effect or non-soy derived isoflavone content. Indeed, the fact that the majority of these diets with such seemingly aberrant isoflavone profiles did not include soy in their list of ingredients, suggests that the source of daidzein may be non-soy plant material. It is possible that the observed differences in ratio and content of isoflavones between domestic cat diets in North America (Court and Freeman 2002) and New Zealand (this study) arise from variations in raw ingredients, strain of soybean, processing treatments, or simply analytical or methodological disparities. Furthermore, the variability evident within and between the categories of domestic cat diets evaluated in this study may also be explained by such factors.

Similar to the findings of Court and Freeman (2002), glycitein was not quantifiable in this study, due to co-eluting compounds within the diet matrix. However, glycitein is usually only a minor component of most soy products, including soy-containing commercial canine foods (Cerundolo *et al.* 2004). As such, glycitein is unlikely to contribute greatly to a physiological effect (Zhang *et al.* 1999). Likewise, biochanin A and formononetin were found to be only minor contributors to the total isoflavone content of domestic cat diets in New Zealand.

If plant materials are used to a greater extent in cheaper diets, this increases the likelihood that those diets will contain greater concentrations of isoflavones. Court and Freeman (2002) reported that the price and isoflavone content of diets were significantly, and negatively, correlated. This is consistent with the results of domestic cat diets in this study. When diets were sorted according to price (regardless of category), the more expensive diets had significantly lower isoflavone contents than the cheaper diets. In contrast, when assessed according to category of diet, moist domestic cat diets containing soy were not only more expensive than those without soy, but they also contained higher concentrations of isoflavones. However, since this category contained only seven diets, all of which were produced by one manufacturer, it is likely that this finding is an artefact of sampling bias.

In clinical trials, Barnes (2003) recommended a limit of 2 mg phytoestrogen/kg BW/day as being safe for humans based on changes in clinical examination parameters. However, significant physiological perturbations have been observed at doses below this limit in other species (Whitten and Patisaul 2001). Within diets assessed in this study, a noteworthy proportion (26/138; 19%) of domestic cat diets contained isoflavones in concentrations capable of meeting or exceeding the 2 mg/kg BW safe exposure threshold. Although consumption of domestic cat diets in the moist meat-only category would be unlikely to expose cats to doses near this threshold, over one quarter of all dry diets evaluated were capable of exceeding that dose, based on estimates of daily intake on a calorific basis. Furthermore, a substantial number of diets contained isoflavones in concentrations capable of providing doses 2 – 4 times greater than this threshold.

The high isoflavone content found in a significant proportion of domestic cat diets reported here suggests that these felids are exposed to physiologically relevant concentrations of isoflavones. The capacity of felids to detoxify isoflavones via hepatic glucuronidation has not yet been established. Although certain isoforms of glucuronosyl transferase, utilised by other mammalian species to glucuronidate exogenous compounds, are deficient or of low activity in felids (Court and Greenblatt 2000), it remains to be

determined whether other detoxifying metabolic pathways for isoflavones are active in felids. Nonetheless, physiological effects of isoflavone exposure, ranging from 1 mg/kg BW – 3 mg/kg BW, via commercially prepared diets have been reported in felids (Setchell *et al.* 1987ab; White *et al.* 2004).

2.2. Experiment Two: Captive Cheetah Diet Analyses

2.2.1. Aim

The aim of this study was to identify and quantify the concentration of dietary isoflavones, genistein and daidzein in diets fed to captive cheetahs, obtained from international zoological facilities.

2.2.2. Materials and Methods

Chemicals, diet sample preparation and HPLC analysis were performed as previously described in Section 2.1.2. No LC-MS was performed on these diets. Only genistein, daidzein, biochanin A and formononetin were quantified, due to the problems encountered previously in the separate elution of glycitein under the analytical conditions employed.

2.2.2.1. *Diets*

Dietary information was obtained from zoological facilities registered as holding cheetah in South Africa, New Zealand and North America (ISIS 2002). Facilities reporting the feeding of commercially-prepared diets to their cheetahs were requested to provide a sample (approximately 100 g, as fed) of diet. Fresh samples were couriered from international institutions and freeze dried, finely ground and thoroughly mixed upon receipt. Samples were stored at -80°C prior to analysis. A total of 27 samples were received and assayed. Participating South African institutions included South African National Zoological Gardens; Cheetah Outreach, Cango Wildlife Ranch, SA Lion Park,

De Wildt Cheetah and Wildlife Trust, Hartebeesport Dam Snake and Crocodile Park, Kyron Laboratory and Hoedspruit Research and Breeding Centre for Endangered Species. Institutions providing samples from the USA were the Wildlife Conservation Society, Jackson Zoo, Montgomery Zoo, Cincinnati Zoo and Botanical Gardens and Mitchel Kalmanson. Participating New Zealand institutions were Auckland Zoo, Wellington Zoo and Orana Wildlife Park.

Dry matter (DM) was determined following oven-drying samples at 105°C for 12 h.

2.2.2.2. *Statistical Analyses*

The diets were classified according to DM content, or intended use. Diets containing between 25% and 50% DM were classified as semi-moist, and those containing more than 90% DM were classified as dry. No diets containing either less than 25% DM or between 50 and 90% DM were received. Based on their intended use, diets were also classified as milk replacers, feline or canine diets, zoo-specific diets, or supplements. Any diets found to contain isoflavones above the detection limit of the assay (1.56 μg/g DM) were termed 'isoflavone-positive'. Values are expressed on a DM basis.

2.2.2.2.a. *Estimated isoflavone intake*

Daily isoflavone intake of adult captive cheetahs was estimated for animals where the test diets constituted the sole-source of nutrition. The isoflavone intake was calculated from the metabolisable energy (ME) content, as follows:

$$\text{Daily isoflavone intake (mg/kg BW)} = \frac{\text{Isoflavone content of the diet (mg/kcal ME)}}{\text{Daily ME requirement of the cheetah (kcal/kg BW)}}$$

Where the daily ME requirement of captive adult cheetahs was assumed to be approximately 50 kcal/kg BW (Dierenfeld 1993) and ME content of the diet calculated using modified Atwater conversion factors (protein x 3.5, fat x 8.5 and carbohydrate x 3.5).

The daily isoflavone intake for cheetah cubs consuming a milk replacer diet was calculated as follows:

$$\text{Daily isoflavone intake (mg/kg BW)} = \frac{\text{Isoflavone content of the diet (mg/kcal ME)}}{\text{Daily DM intake of the cub (kcal/kg BW)}}$$

Where the DM intake of hand-reared cheetah cubs was determined by Bell *et al.* (2008, unpublished) from the feed intake of 18 hand-reared cubs fed one of the milk replacers analysed here.

Residual data were tested for normality with the Anderson-Darling test and found not to be normally distributed. Diets of each category were compared for isoflavone content using the Kruskal-Wallis test statistic (H) for significant difference. The same procedure was used to compare diets with previous findings for domestic cat diets (section 2.1.3.1.). Median isoflavone exposure was compared between cheetahs and domestic cats (Section 2.1.3.2.) using the Kruskal Wallis test for significance. All statistical analyses were performed using MINITAB (version 15, Minitab Inc., PA, USA).

2.2.3. Results

Sixteen international facilities responded to the survey and provided a total of 27 diets. Of all respondents, over half (9/16) reported utilising, or having previously utilised, some form of commercially-prepared diets when feeding captive cheetahs. All five North

American facilities utilised commercially-prepared diets whilst 75% (6/8) of the South African facilities utilised commercially-prepared diets. No New Zealand facilities utilised commercially-prepared diets.

Three supplements, three milk replacer powders, six semi-moist diets and 12 dry diets were assayed. Of the 27 diets assayed only six diets were specifically manufactured for zoo-held carnivores, and all of these were classified as semi-moist from their dry matter content (herein referred to as zoo-specific diets). The rest of the diets were commercially available dry canine or feline diets (herein referred to as "dry") which were reported by the donating facilities to be used only as "extenders" of meat-based diets for captive non-domestic felids. Therefore, these dry diets did not contribute solely to the nutrition of these animals.

2.2.3.1. *Frequency of occurrence of isoflavones in captive cheetah diets*

The number of diets found to contain detectable isoflavone concentrations was determined (see Table 2.3). Twenty two diets (81%) were found to contain detectable amounts of isoflavones. Daidzein was the most commonly detected isoflavone, being present in all of the isoflavone positive diets (22/27), followed by genistein which was found in 20/27 (74%) of the diets assayed. Biochanin A was detected in 7/27 (26%) of diets but formononetin was detected in only one diet (4%). Genistein was present as the isoflavone of the greatest concentration in 19/27 (70%) of tested diets, while daidzein was the most abundant isoflavone in 8/27 (30%) of diets.

Of the zoo-specific diets, 4/6 (67%) were found to contain daidzein, whilst only 3/6 (50%) contained genistein. All dry diets contained daidzein and genistein. One of the three milk replacer diets did not contain either daidzein or genistein, whilst the other two contained both forms of isoflavone. None of the supplements assayed contained genistein, but one was found to contain daidzein.

Category of diet	n	Genistein-positive diets (%)	Daidzein-positive diets (%)
Zoo-specific	6	50	68
Dry	15	100	100
Milk replacer	3	67	67
Supplement	3	0	33

Table 2.3. Percentage of captive cheetah diets within defined categories that contained detectable amounts of genistein and daidzein.

2.2.3.2. *Isoflavone content of captive cheetah diets*

The isoflavone content of the 27 tested diets was determined, and is shown in Table 2.4. Dry, supplement and zoo-specific diets were found have a right-skewed distribution (skewness statistics 1.16, 1.73 and 1.09, respectively; kurtosis values -0.23, immeasurable and -0.74, respectively). Milk replacer diets displayed a left-skewed distribution (skewness statistic -1.76 and kurtosis statistic immeasurable). No significant differences in isoflavone content were detectable between diet categories ($H = 6.58$, $p > 0.05$).

Category of diet	n	Genistein content	Daidzein content	Total content
			mg/kg DM	
Zoo-specific (semi-moist)	6	0.12 (0 – 72.3)	1.72 (0 – 58.0)	6.02 (0 – 137)
Dry	15	8.63 (2.15 – 74.5)	10.8 (2.30 – 58.4)	17.4 (4.45 – 133)
Milk replacer	3	3.13 (0 – 138)	37.0 (0 – 44.5)	175 (0 – 183)
Supplement	3	0 (0)	0 (0 – 1.75)	0 (0 – 1.75)

Table 2.4. Median (minimum, maximum) genistein, daidzein and total isoflavone content of diets within defined categories.

The median total isoflavone content across all diets was 15.6 mg/kg DM. Median genistein content was 3.49 mg/kg DM and the median daidzein content was 5.78 mg/kg DM. Only one diet contained detectable amounts of formononetin and this was a zoo-specific diet containing low concentrations of genistein and daidzein (2.97 mg/kg DM and 2.04 mg/kg DM, respectively), but a relatively high concentration of formononetin (16.6 mg/kg DM), and no detectable biochanin A. Two of the four biochanin A-positive, dry diets contained this isoflavone in greater concentrations than that of genistein or daidzein. A similar trend was observed for biochanin A-positive diets in the zoo-specific and milk replacer diet categories. Of the isoflavone-positive diets, total isoflavone content ranged from 1.75 – 182 mg/kg DM.

Since only the zoo-specific diets and milk replacers were reported as being used as the sole source of nutrition for captive cheetahs these diets were evaluated in more detail. The isoflavone contents of zoo-specific diets (Table 2.5) had a median isoflavone content of 6.02 mg/kg DM. Total isoflavone content of zoo-specific diets ranged from 0 – 137 mg/kg DM. Genistein was detected in concentrations up to 72.3 mg/kg DM and daidzein up to 58.0 mg/kg DM.

Isoflavones were not detectable in two of the six (33%) zoo-specific diets. Only one zoo-specific diet contained appreciable concentrations of genistein or daidzein, where the majority of diets in this category contained isoflavones close to the detection limit of the assay (see Table 2.5). The only zoo-specific diet that contained significant concentrations of isoflavones was also the only diet of this category to list soy as an ingredient.

Two of the three milk replacers used for hand-rearing captive cheetah cubs contained detectable concentrations of isoflavones. One milk replacer contained 175 mg/kg DM total isoflavones, comprised primarily of biochanin A (135 mg/kg DM), with genistein and daidzein present in much lower concentrations (3.13 mg/kg DM and 37.0 mg/kg DM,

respectively). The other replacer contained the highest concentration of isoflavones of all milk replacers (183 mg/kg DM total isoflavones), with 138 mg/kg DM genistein and 44.5 mg/kg DM daidzein. The third milk replacer did not contain any detectable isoflavones.

Diet	Genistein	Daidzein	Total[1]
		mg/kg DM	
Zoo-specific 1	0.25	1.40	3.59
Zoo-specific 2	72.3	58.0	137
Zoo-specific 3	BD	2.61	8.45
Zoo-specific 4	2.97	2.04	21.7
Zoo-specific 5	ND	ND	ND
Zoo-specific 6	BD	BD	BD
Milk replacer 1	138	44.5	183
Milk replacer 2	BD	BD	BD
Milk replacer 3	3.13	37.0	175

Table 2.5. Dietary isoflavone content of zoo-specific commercially prepared diets fed to captive cheetahs. [1] the sum of genistein, daidzein, formononetin and biochanin A content. BD = below the assay detection limit (1.56 μg/gDM). ND = no peak detected.

2.2.3.3. *Isoflavone intake by captive cheetahs*

Only zoo-specific diets were utilised for estimating isoflavone intake of captive adult cheetahs since institutions reported that all other diets (dry diets and supplements) consumed by adult cheetahs did not provide the sole source of nutrition for these animals. The estimated dietary isoflavone intakes of an adult cheetah consuming the zoo-specific diets tested here are shown in Table 2.6.

Overall median isoflavone intake across all the tested diets consumed by adult cheetahs was 0.23 mg/kg BW. The median estimated intake of isoflavones by adult captive cheetahs was highest for the dry diets (0.73 mg/kg BW), compared to zoo-specific diets (0.07 mg/kg BW) when calculated from the estimated energy requirement of the adult cheetah. Adult cheetahs fed the zoo-specific diet # 2 would have the highest isoflavone intake (1.47 mg/kg BW) whilst the remaining 5 zoo-specific diets would provide isoflavones in concentrations lower than 0.26 mg/kg BW.

Diet	Genistein intake	Daidzein intake	Total isoflavone exposure
		mg/kg BW	
Zoo-specific 1	0.00	0.02	0.04
Zoo-specific 2	0.77	0.62	1.47
Zoo-specific 3	0.00	0.03	0.10
Zoo-specific 4	0.04	0.02	0.26
Zoo-specific 5	0	0	0
Zoo-specific 6	0	0	0
Milk replacer 1	3.20	1.03	4.24
Milk replacer 2	0	0	0
Milk replacer 3	0.07	0.86	4.06

Table 2.6. Dietary isoflavone intake predicted for captive cheetahs[1] consuming commercially prepared zoo-specific diets and cubs[2] reared on commercially prepared milk replacers.

[1]Daily isoflavone intake for the adult cheetah was calculated from the isoflavone content of the diet expressed on an energy basis and the energy requirement of the adult cheetah (50 kcal/kg BW) (Dierenfeld 1993).

[2]Daily isoflavone intake for the cheetah cub was calculated from the isoflavone content of the diet expressed on DM basis and the measured daily DM intake of hand-reared cheetah cubs (mean 23.21 g/kg BW) (Bell *et al.* 2008, unpublished).

The calculated median exposure of adult cheetahs consuming either dry, or zoo-specific diets analysed here (0.23 mg/kg BW) was double that reported previously for domestic cats (0.11 mg/kg BW) although this difference was not statistically significant ($p > 0.05$). Cheetah cub exposure from milk replacers ranged from 0 (milk replacer #2) to 4.24 (the latter via milk replacer #1).

Overall, of the zoo-specific diets assayed, 1/6 (17%) was predicted to provide cheetahs with a total daily isoflavone intake greater than 1 mg/kg BW whilst 2/3 (67%) milk replacers were predicted to provide cheetah cubs with a total daily isoflavone intake greater than 2 mg/kg BW.

2.2.4. Discussion

Isoflavones were detected in the majority of diets used to feed captive cheetahs. Over 80% of the diets evaluated were found to contain at least one of the four isoflavones investigated. This is considerably higher than the 46% occurrence in domestic cat diets (Court and Freeman 2002) or 57% of canine diets (Cerundolo *et al.* 2004) available in North America, but is similar to the frequency of isoflavone-occurrence in commercially-prepared feline diets available in New Zealand (> 75%) (Section 2.1.3.). The representativeness of captive cheetah diets tested here is unclear as a dietary survey of all facilities listed as holding cheetahs was beyond the scope of this thesis. However, the zoo-specific diets tested were considered by the donating facilities to represent the majority of diets available to zoos in North America.

As in the previous study (Section 2.1.3), daidzein was the most frequently detected isoflavone, followed closely by genistein and biochanin A and formononetin contributed only minor amounts towards the total isoflavone content of most diets assessed. One milk replacer contained a far greater concentration of biochanin A than genistein or daidzein.

Biochanin A is a derivative of genistein such that demethylation of genistein during processing of this diet may be responsible for this finding.

The six zoo-specific diets assayed which provided the sole-source of nutrition for captive cheetahs exhibited a lower incidence (68%) of isoflavone-positive diets than New Zealand domestic cat diets (Section 2.1.3.). However, these diets were still more likely to contain isoflavones than the companion animal diets assayed in North American studies (Court and Freeman 2002; Cerundolo *et al.* 2004). Despite the relatively small sample size, these finding suggest isoflavone intake for captive cheetahs may be significant. Epidemiological analysis of the intake of these isoflavone-containing diets and captive cheetah morbidity was beyond the scope of this thesis.

The median daidzein, genistein and total isoflavone contents of all analysed captive cheetah diets (5.78, 3.49 and 15.56 mg/kg DM, respectively) were not significantly different from that of the domestic cat diets (3.21, 0.0 and 5.98 mg/kg DM, respectively) analysed in Section 2.1.3 (Figure 2.2). When only isoflavone-positive diets were included in the analyses, the median daidzein, genistein and total isoflavone content of adult captive cheetah diets (9.43, 6.64 and 19.50 mg/kg DM, respectively) also did not differ from those of domestic cat diets (Section 2.1.3.) (4.99, 2.80 and 8.86 mg/kg DM, respectively) ($p > 0.05$). The difference between daidzein, genistein and total isoflavone concentrations in diets in the zoo-specific category and domestic cat diets analysed in Section 1.2 also failed to achieve statistical significance.

The estimated intake of adult cheetahs was based on the calculated energy requirement of the adult cheetah reported by Dierenfeld (1993), which assumed that cheetahs will consume sufficient food to meet their metabolic energy requirement. Estimates of metabolisable energy intake, and therefore isoflavone intake calculated here, indicate the mean daily feed intake for a 47 kg adult cheetah to be 1.11 kg (as-fed), which is similar to the 1.14 kg per day reported by Bechert *et al.* (2002), and only slightly less than the 1.32

kg reported by Dierenfeld (1993) for adult captive cheetahs consuming one of the zoo-specific diets assayed here. Given the similarity between calculated food intake estimates reported here and intakes determined by other workers, this method of calculating food intake for adult cheetah appears to have been suitable. Accurate reports of energy intake and milk replacer intake for hand-reared cheetah cubs are scarce. Bell *et al.* (unpublished) determined dry matter intake for 18 cheetah cubs, over 4 breeding seasons and provides the most dependable database of cub intakes. In the study of Bell *et al.* (unpublished) cubs consumed one of the milk replacers analysed here and were found to have growth rates comparable to previous reports for hand-reared cheetah cubs (Beekman et al.. 1991; Grant 2005; Schumann and Schumann 1995) which validates the use of this data. The estimated isoflavone intake of cubs, if reared on the milk replacers analysed in this study, was higher than observed in any other diet category, with intakes up to 4.06 mg/kg BW.

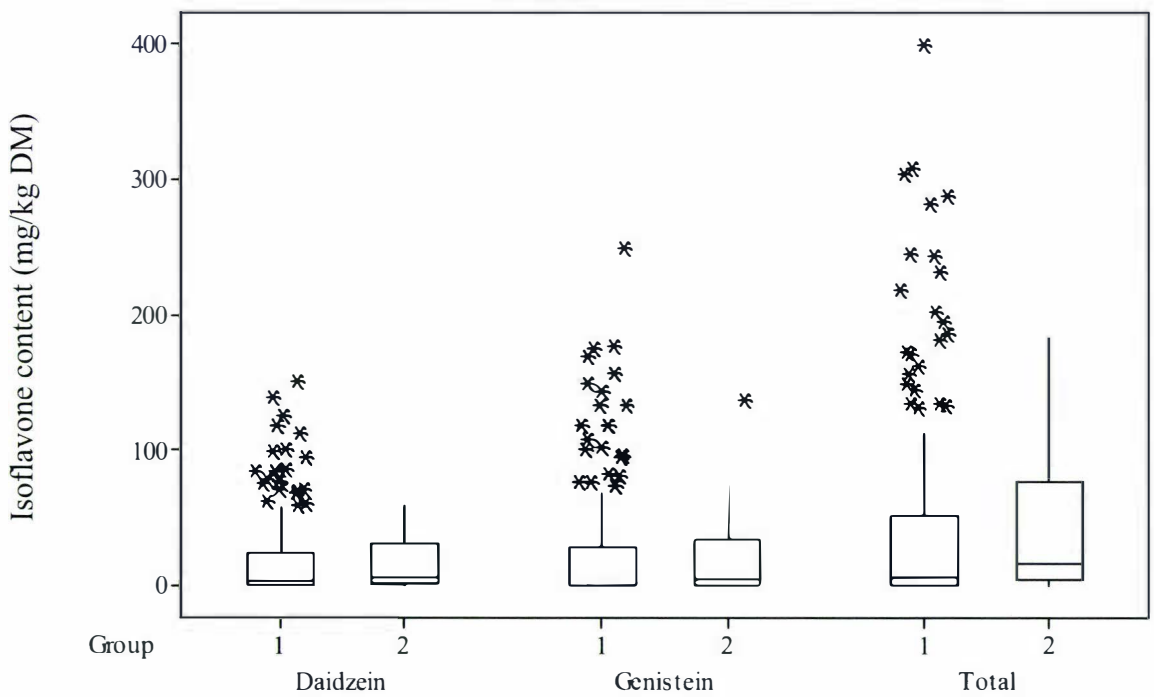


Figure 2.2. Box plot of the daidzein, genistein and total isoflavone content (mg/kg dry matter) of domestic cat (1) and captive cheetah (2) diets.

The outliers are indicated by * whilst the upper whisker extends to the highest data value within the upper limit (upper quartile + 1.5). The top of the box represents the third

quartile (75% quartile), and the bottom of the box represents the lower quartile (25% quartile). The median is represented by the line within the box. The lower whisker extends to the lowest value within the lower limit (lower quartile + 1.5).

Diets consumed by captive cheetahs contained isoflavones with a median concentration of 15.6 mg/kg DM and would provide cheetahs with a median isoflavone dose of 0.23 mg/kg BW. Within the diets consumed by adult cheetahs, only one zoo-specific diet approached the 2 mg/kg BW exposure threshold (zoo-specific # 2) set for human clinical trials (Barnes 2003). Other zoo-specific diets are known to be available on the market but could not be obtained for this study. Given that soy is known to be an important ingredient in some of these diets, it is possible that isoflavone exposure from these other diets may be significant and further investigation is warranted.

One zoo-specific diet evaluated in this study, which is recommended by the American Zoological Association and reported as a "complete and balanced" diet for captive cheetahs (Cheetah Conservation Fund 2000) was found to contain the highest concentration of isoflavones. This diet (zoo-specific # 2) was calculated to provide captive cheetahs with 1.47 mg/kg BW total isoflavones per day, when fed as the sole source of nutrition to the animals. This is at least 7 times higher than the median exposure achievable for domestic cats (0.11 mg/kg BW) predicted in Section 2.1.3.2, but below the level of exposure known to induce biological perturbations in domestic cats (White *et al.* 2004; Cave *et al.* 2007b). Interestingly, this value is identical to the median exposure of domestic cats predicted from feline diets containing soy, and is very similar to that estimated from data presented for the same diet assayed by Setchell *et al.* (1987a).

However, the minimum effective dose has not been determined in felids for more than one physiological parameter (Cave *et al* 2007b). Consequently, further study is warranted. The dry diets fed to captive cheetahs were all commercially-available feline or canine foods but were not the sole source of nutrition. Yet, given the high incidence and relatively high concentrations of isoflavones within this diet category, even

intermittent feeding of these diets may expose captive cheetahs to noteworthy doses of isoflavones.

Previous studies (Dierenfeld 1993; Bechert *et al.* 2002) have suggested that zoo-specific diet # 1 is the most popular captive cheetah diet in surveyed North American institutions (used by 10 of 13 facilities surveyed in the Dierenfeld (1993) study). This diet was determined to contain negligible concentrations of isoflavones, indicating that isoflavone exposure to captive cheetahs and other non-domestic felids is not likely to be a concern in institutions utilising this diet. A more extensive survey of international institutions is warranted to determine the utilisation of zoo-specific diet # 2, and to assess the isoflavone content of other diets utilised for feeding cheetahs.

Of particular concern is the high isoflavone content in one milk replacer (milk replacer # 1), used to hand-rear captive cheetahs. The absolute isoflavone content of this diet was comparable to those at the higher end of the scale of diets fed to adult cheetahs. Yet, given the higher daily feed intake of growing animals, this diet would expose cheetah cubs to doses as high as 4.2 mg/kg BW. This dose is approximately 4 times higher than the highest exposure predicted for adult cheetahs. Another milk replacer (# 3) also contained high concentrations of total isoflavones (up to 4.1 mg/kg BW) although this was predominantly composed of biochanin A, for which relatively little is known in regards to its potential for physiological perturbation. Exposure to genistein and daidzein from milk replacer # 3 is predicted to be low, with genistein and daidzein exposure reaching levels well below 2 mg/kg BW. Isoflavone exposure during neonatal periods is known to elicit divergent, and often greater, responses than observed in adults (Medlock *et al.* 1995; Hollander 1997; Nagao *et al.* 2001; Souzeau *et al.* 2005) and the high isoflavone content of milk replacer # 1 may, therefore, have consequences for cheetah cubs later in life. Such effects may include modulated puberty onset, impaired fertility, altered fecundity or developmental changes (see Section 1).

2.3. Conclusions

- Isoflavones occurred in 75% of commercially-available domestic cat diets evaluated and 81% of cheetah diets. Total isoflavone concentration ranged from 1.65 – 400 mg/kg DM in domestic cat diets and 4.45 – 183 mg/kg DM in cheetah diets.

- The genistein-to-daidzein ratio was unusually biased towards daidzein in one third of domestic cat diets analysed. This may indicate non-soy derived isoflavones or a processing effect.

- The median predicted daily intake of isoflavones for cats consuming these diets was 0.11 mg/kg BW (maximum 8.13 mg/kg BW) and 0.23 mg/kg BW (maximum 1.47 mg/kg BW) for cheetahs. Cheetah cub exposure via one milk replacer was nearly 4 times greater than that predicted for adult cheetahs.

- The isoflavone concentrations and intakes predicted here are within the range of doses that have been shown to exert significant biological activity in other mammals, in both long- and short-term exposure studies.

- Hence, the dietary content of the evaluated diets, fed to domestic cats and captive cheetahs, may be sufficient to elicit physiological changes. The high daily intake of isoflavones during the neonatal period is of particular concern for cheetah cubs. However, the effects of isoflavone intake by felids are unknown, and consequently investigation of this, as well as the cat's ability to absorb and metabolise dietary isoflavones, warrant further study.

CHAPTER THREE:

Absorption, Excretion and Metabolism of Genistein and Daidzein in Domestic Cats (*Felis catus*)

A pilot study to this experiment was conducted, the findings of which were presented at the 2005 Waltham International Nutritional Sciences Symposium, Washington D.C., USA and published (Appendix 3) as an expanded abstract as: **Bell KM,** Pearce PD, Ugarte CE, Hendriks WH. (2006). Preliminary investigation of the absorption of dietary isoflavones in the domestic cat (*Felis catus*). *Journal of Nutrition* 136: 2004S – 2006S.

3.0. Introduction

Analysis of diets consumed by domestic cats and cheetahs demonstrated that these diets contain isoflavones, often in significant concentrations (see Section 2.1.3 and 2.2.3). The isoflavone genistein is known to cross the intestinal barrier of the domestic cat (Cave *et al.* 2007a) and has elicited physiological changes in other species after oral exposure. The finding that cats are being exposed to isoflavones through the daily consumption of certain diets (Section 2.1.3) indicates that isoflavones may elicit physiological perturbations in this species.

To date, no study has determined the ability of a felid species to absorb, metabolise and excrete isoflavones when consumed at concentrations achievable from dietary sources. One study (Cave *et al.* 2007a) describes the bioavailability and pharmacokinetics of purified genistein in domestic cats when fed at 100 mg/kg BW. However, this dose greatly exceeds even the maximum dose achievable from commercially prepared diets (predicted to be 8 mg/kg BW, see Section 2.1.3.2.). Before the potential physiological impact of dietary isoflavones can be predicted in felids it is important to gain an understanding of the absorption, metabolism and excretion of isoflavones by felids at relevant doses.

The first two of these studies were aimed at determining the metabolites detectable in felid plasma following oral exposure to genistein and daidzein as either a tablet or part of a dietary matrix. Following this a mass balance study was conducted to establish the fraction of dose that was recovered in urine, plasma and faeces. An estimate of apparent absorption was made using a serial blood sampling regime and calculation of total recovered isoflavone present in the blood of cats for the 4 day period following isoflavone exposure. To re-establish terminology, "genistein" and "daidzein" refer to the unbound or aglycone forms of these isoflavones, whereas when present in a conjugated form the type of conjugating moiety is defined (e.g. genistein monosulphate). When the

form of isoflavone is not known (e.g. in the soy extract and dietary matrix) the generic terms "genistein" and "daidzein" are used.

3.1. Experiment One:

Genistein and Daidzein Metabolism in Domestic Cats Following Ingestion of a Single Oral Bolus of Soy Extract

3.1.1. Aim

The capacity for intestinal absorption of genistein in the domestic cat has been established (Cave *et al.* 2007a; Appendix 3) but isoflavone metabolites have not been determined. This study was therefore designed to describe the metabolites of both genistein and daidzein present in the plasma following a single oral bolus of soy extract.

3.1.2. Materials and Methods

3.1.2.1. *Animals*

Six adult male, domestic short-haired cats, with a mean age of 3.75 years (± 0.65) and weighing 4.58 kg (± 0.12) on average, were used in this trial. The trial was approved by Massey University Animal Ethics Committee (2003). The cats were bred and housed at the Massey University Centre for Feline Nutrition, and had been maintained on a commercially prepared diet, assayed to contain no detectable isoflavones, for 4 weeks prior to initiation of the trial. This washout period was estimated to be more than sufficient, based on literature published in other species. Antibiotics had not been administered to any of these cats for 3 months prior to the trial to prevent a disturbance in their normal gut microflora.

3.1.2.1.a. *Pre-trial conditioning/testing*

All cats in the trial had previously been exposed to blood sampling and metabolic cage training, and were acclimatised to frequent handling and humane manipulations. Discrete meals (twice daily) were fed during the five day period prior to the onset of the trial, in order to adapt cats to the feeding regime that would be employed during the study. The packed cell volume of each cat was confirmed to be within normal range prior to the study. This was measured from a droplet of blood obtained from a jugular vein blood sample of each cat. A capillary tube was filled with blood and a wax plug inserted in one end. The capillary tubes were then centrifuged in a purpose-designed centrifuge and the ratio of red blood cells-to-serum was measured.

In order to maintain the patency of indwelling catheters it was necessary to flush the catheters regularly with heparinised saline. To avoid dilution of collected blood samples with this flushing agent, blood containing heparin was removed from the indwelling catheter (described in Section 3.1.2.3.a) prior to the collection of each blood sample. A preliminary experiment was performed to ascertain how much blood must be removed before an appropriate blood sample, representative of whole-body circulation, was obtained. A series of consecutive blood samples (in 0.5 ml aliquots) were withdrawn from the catheter of one cat, following flushing with heparinised saline. Packed cell volume (PCV) values from each blood sample was measured, and compared to the normal PCV, as measured from a single sample obtained by venipuncture of a non-catheterised vein in the same cat. A total of 4 ml of blood was required before a normal packed cell volume was achieved in samples from the catheterized vein. This volume was withdrawn prior to obtaining each sample during the trial, but was infused back into the cat after collecting each sample, to minimize blood loss in each animal.

The plasma-sampling regime was calculated to ensure less than 10% total blood volume was removed during the trial, as per the maximum safe limit for serial blood sampling within a 24 hour period (Diehl *et al.* 2001).

3.1.2.1.b. *Trial conditions*

For the duration of the trial, cats were individually housed in polythene plastic metabolism cages (0.8 x 0.8 x 1.1 m) equipped with an elevated rest area, feed and water bowl area and litter tray. The cats were exposed to natural light cycles and provided with *ad libitum* access to water. A nutritionally complete and balanced basal diet (meeting all NRC (1986) requirements for maintenance in the domestic cat) was provided as two discrete meals. The total amount of diet offered was calculated as sufficient to meet the metabolisable energy (ME) requirements of an inactive cat, in the maintenance life-stage (ME = 70 kcal/kg body weight/day, NRC 1986). The proximate composition of the base diet is detailed in (Table 3.1).

	Dry Matter (DM)	Crude Fat (%)	Crude Protein (%)	Carbohydrate (%)*
Base diet	17.73	25.2	35.6	29.2

Table 3.1. Proximate composition of basal diet used in the experiments described in Section 3.

*Calculated by difference

3.1.2.2. *Isoflavone dose*

Isoflavones were administered as a soy extract tablet (Phytolife1®: One a Day, Healthy Living, Palmerston North, New Zealand), reported to contain isoflavone glycosides from 26.8 g dry soy bean seed (*Glycine max*). Analysis (according to methodology described in Section 2.1.2.) determined these tablets to contain 10.17 mg genistein and 13.12 mg daidzein (as-fed), reported as aglycone equivalents. Analysis without prior hydrolysis determined the tablets to be comprised of 0.35 mg genistein aglycone and 9.82 mg conjugated genistein. Daidzein aglycone accounted for 0.73 mg of the dose administered, with the remaining 12.39 mg containing conjugated daidzein. A total of 37.58 µmol genistein and 48.48 µmol daidzein was administered to each cat, which equated to a mean dose of 2.22 mg genistein/kg BW (± 0.06) and 2.86 mg daidzein/kg BW (± 0.08), and a

total isoflavone dose of 5.08 mg/kg BW (± 0.13). All absorption and excretion parameters are normalised to individual dose for each cat prior to averaging.

3.1.2.3. *Plasma collection*

3.1.2.3.a. *Catheter placement*

All surgical and bandaging equipment was provided by Southern Veterinary Supplies, Palmerston North, New Zealand, unless otherwise specified. Indwelling catheters were placed in a jugular vein of each cat 24 h prior to the onset of the trial. To facilitate catheter placement, anaesthesia was induced with propofol (6 – 8 mg/kg BW, intra-venous) and a surgical concentration of anaesthesia maintained by halothane gas administered via an endotracheal tube (size 3.5). The cat was positioned in dorso-ventral recumbancy, with the neck outstretched and a jugular vein palpable. An area of approximately 5 cm^2 over the jugular vein was clipped of hair and prepared aseptically for venipuncture, using chlorhexidine surgical scrub and methylated spirits. A 20 gauge x 8 cm indwelling jugular catheter (Shoof International, Auckland, New Zealand) was then placed in a jugular vein of each cat using a modified Seldinger (Seldinger 1953) technique and inserted approximately immediately cranial to the heart.

The catheter was then flushed with sterile normal saline (0.9% saline) and then again with heparinised saline (10 IU heparin/ml normal saline). An extension set (pre-filled with heparinised saline), and luer-lock port were then attached to the catheter butterfly port, and the insertion site cleaned of blood and dried, prior to suturing the catheter in place using 2 sutures (3/0 Supramid). The total volume of catheter and extension set was calculated as 0.7ml. Betadine ointment was applied to the insertion site, and two gauze swabs were rolled up and placed under the extension set hub to prevent rubbing on the cat's face or neck. The area was bandaged with Easifix, followed by Vet-wrap (see Figure 3.1). The cat's back legs were hobbled with Elastoplast to prevent scratching at the neck bandage.

The cats were allowed to recover in small transport cages with padded sides until standing and alert, after which time they were placed in metabolism cages. A 24 hour recovery period was allowed between catheter placement and the first sampling period, to maximise elimination of anaesthetic agents from circulation. During this time the catheters were maintained by thrice daily flushing with 1 ml heparinised saline (10 IU/ml). A 0.7 ml heparin lock (50 IU/ml) was used during the 12-hour overnight period and this was removed first thing the following morning.

3.1.2.3.b. *Blood collection technique*

Blood samples were withdrawn from the jugular of each cat via the indwelling catheter using a "two-syringe" technique. A sterile syringe was first used to withdraw 4ml of heparinised blood from the catheter, in order to clear the catheter ready for sampling. A second syringe was then used to collect 1.5ml of blood (Figure 3.1), which was transferred immediately into a heparinised vacutainer. The contents of the first syringe were then re-administered in order to minimise blood loss, before the catheter was flushed with 1 ml heparinised saline (10 IU/ml). Sterility was maintained at all points during sampling. Within 10 min of collecting the sample, the blood was centrifuged for 10 minutes at 3000 rpm and the plasma transferred to labelled vials. All plasma samples were stored at -20°C prior to analysis.

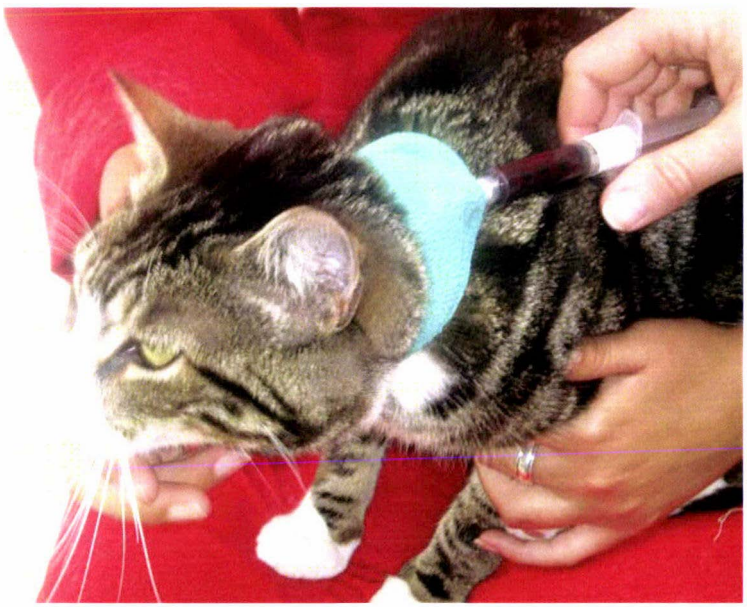


Figure 3.1. Blood collection from an indwelling catheter in the jugular vein of a domestic cat

3.1.2.4. *Isoflavone administration and sampling schedule*

The isoflavones were administered as a soy extract tablet, and the time of administration recorded as time 0. Serial blood samples were collected thereafter. In order to mimic isoflavones consumed as part of a dietary matrix, the tablets were administered post-prandially. Cats were fasted for 18 h prior to administration of the tablet to maximise consumption of the meal. The tablet was placed at the back of the throat of each cat, 5 min following the offering of the meal, and throat massage was applied until the tablet was swallowed. A baseline blood sample was collected into a heparinised vacutainer immediately prior to administering the tablet. Blood samples were then collected at 2, 4, 6, and 12 h post-isoflavone ingestion. After the final sampling period the catheters were removed. Catheter insertion sites were re-bandaged for a further 24 hours to ensure healing, after which time bandages were removed and cats were returned to normal housing conditions.

3.1.2.5. *Plasma analysis*

A liquid chromatography (LC)-electrospray (ESI)-mass spectrometry(MS)/MS assay was used to measure genistein, daidzein and their metabolites in plasma. Pilot study (Appendix 3) samples were screened for all known metabolites of genistein and daidzein (see Section 1.3.2) and only these were assessed in all subsequent studies. Standards containing isoflavone sulphates (genistein 4'-monosulphate, daidzein 4'-monosulphate, equol 7-monosulphate, equol 4'-7-disulphate, daidzein 4'-7-disulphate) were prepared. The isoflavone sulphate standards were donated by Dr. Nigel Botting (Department of Chemistry, St. Andrew's University, Fife, UK). All other isoflavones, genistein, daidzein and equol were obtained from LC-Laboratories (Woburn, MA, U.S.A). Solvents were supplied by Fisher (Norcross, GA, U.S.A).

Samples were prepared by the protein precipitation method such that samples were diluted 5-fold with methanol containing 0.1% acetic acid. Liquid chromatography was carried out on a reversed-phase phenyl-hexyl column (100 x 2.0 mm internal diameter; Phenomenex, CA, USA). The mobile phase consisted of solvent A (water, containing 10 mM ammonium acetate) and solvent B (acetonitrile, containing 10 mM ammonium acetate) with a flow rate of 0.2 ml/min. Liquid chromatography-MS/MS analysis was performed using a system consisting of a model SIL-HT refrigerated Shimadzu autosampler (Shimadzu Scientific Instruments; Columbia, MD, USA), and an API 4000 (Applied Biosystems/MDS Sciex; Concord, Ontario, USA), which was used for quantitation and identification. Nitrogen was used as the nebulising gas. Mass-to-charge ratios (m/*z*) used for MRM analysis were 333/117 (daidzein 4'-monosulphate), 413/333 (daidzein 4'-7-disulphate), 349/269 (genistein 4'-monosulphate), 401/321 (equol 4'-7-disulphate) and 321/121 (equol monosulphate). The quantification of isoflavone sulphates was based on a calibration curve of each isoflavone sulphate in the concentration range of 10-10,000 nM.

3.1.2.6. *Plasma data interpretation*

All values are expressed as mean (± SEM) unless otherwise stated. The maximum plasma concentration (C_{max}), and time of achieving this concentration (T_{max}) were determined using non-compartmental modelling software (WinNonLin Professional, v 4.1. Pharsight Corp, NC, USA).

3.1.3. Results

Unbound daidzein and genistein were detected in the plasma of five out of six cats, whilst neither genistein nor daidzein were found in one cat in their unbound forms. No glucuronide metabolites were detected. The metabolites daidzein monosulphate, daidzein disulphate, and genistein monosulphate were detected in the plasma of all cats. Both unbound genistein and daidzein were no longer detectable in plasma at 12 h post-isoflavone ingestion although all other metabolites were still detectable at this final sampling point.

	Tmax (h)	Cmax (μmol/L)
Unbound genistein	2.40 (± 0.09)	0.09 (± 0.04)
Unbound daidzein	2.40 (± 0.69)	0.10 (± 0.10)
Genistein monosulphate	3.00 (± 0.68)	14.43 (± 4.02)
Daidzein monosulphate	4.33 (± 1.58)	6.66 (± 1.67)
Daidzein disulphate	4.67 (± 1.52)	3.27 (± 0.73)

Table 3.2. Pharmacokinetic parameters for cats administered with 37.58 μmol genistein and 48.48 μmol daidzein (2.22 mg genistein/kg BW and 2.86 mg daidzein/kg BW). Values are expressed as mean (± SEM).

Equol was only detected at the final sampling point (12 h post-dose) in one cat and in this cat it occurred as a monosulphate. No other forms of equol were detected in any cat. Dihydrodaidzein and *O*-DMA were not detected in any cat. The C_{max} and T_{max} are shown in Table 3.2.

The proportion of genistein occurring in the plasma as a conjugate increased from a mean of 99.41% (± 0.002) at the 2 h post-dose, to 99.98% (± 0.003) at 12 h post-dose. The proportion of daidzein occurring in the plasma as a conjugate increased from a mean of 99.03% (± 0.003) at 2 h post-dose, to 99.93% (± 0.000) at 12 h. No significant difference was detectable between the extent of conjugation observed for genistein or daidzein.

3.1.4. Discussion

Both genistein and daidzein appeared in detectable concentrations in the plasma of the domestic cat following ingestion of a soy extract tablet, confirming the ability of the cat to absorb these isoflavones.

Conjugation to glucuronic acid is thought to be the principal pathway involved in the inactivation and subsequent excretion of isoflavones in other species (Setchell 1995; Jäger *et al.* 1997; Kurzer and Xu 1997; Andlauer *et al.* 2000ab) and serves to reduce the binding affinity of isoflavones for the oestrogen receptors (Kinjo *et al.* 2004). The low activity of glucuronidation pathways in cats has previously been thought to increase the susceptibility of cats to the biological activity of isoflavones (Setchell *et al.* 1987a; Court and Freeman 2002). The lack of detectable glucuronide conjugates detected in the plasma of domestic cats here is in agreement with previous findings of the poor glucuronidation capacity in this species (Hartiala 1955; Robinson and Williams 1958).

However, the findings of this study provide the first evidence that domestic cats are capable of significant metabolism of ingested isoflavones. Indeed, the bulk of detected

isoflavones found to occur in feline plasma were present as sulphate conjugates, with only minimal proportions found in an unbound form. Hence, felids appear to have a comparable conjugation capacity to other species, and may be no more or less susceptible to the physiological changes observed following isoflavone exposure in other species. There was also no detectable temporal fluctuation in the degree of conjugation for either genistein or daidzein although studies with increased frequency of sampling would be necessary to confirm this.

The metabolite profile of feline plasma reported here indicates that phase II metabolism of genistein and daidzein involves only conjugation with sulphate moieties. Plasma concentrations of unbound genistein and daidzein were minor (<1% of the total plasma isoflavone content), compared to that of their respective sulphate conjugates. This has also previously been shown for unbound forms in other species (Setchell *et al.* 2001; Bloedon *et al.* 2002; Soucy *et al.* 2006). It is likely that the apparent deficiency in glucuronide conjugating pathways in the cat is compensated for by efficient sulphation mechanisms. Furthermore, at T_{max}, over 99% of the isoflavones detected were conjugated, indicating that the conjugation process is sufficient to keep up with the rate of absorption.

Some isoflavone-specific differences were observed. The only conjugate detected of genistein was the monosulphate, whereas daidzein was shown to be conjugated to either one or two sulphates. However, the disulphate conjugate of daidzein was present in lower concentrations than that of its monosulphate at all time points. Genistein monosulphate appeared in higher concentrations than either daidzein disulphate or monosulphate, at all time points, which is consistent with previous reports in human plasma (Setchell *et al.* 2003ab) and does not reflect the relative concentrations of each isoflavone provided in the dose.

Contrastingly, the concentration of unbound genistein and daidzein were comparable at all time points, indicating equivalent potential biological exposure to these more active forms. This highlights the importance of identifying discrete metabolites, since reporting only total equivalents may unjustly bias plasma concentrations towards genistein, due to the higher concentrations of genistein monosulphate. Furthermore, the peak plasma concentration of unbound genistein was over 100 times lower than that of genistein monosulphate.

The only other detectable metabolite of daidzein was equol monosulphate. However, this metabolite of daidzein was only detected in one cat, at one time point. This individual's unique, albeit limited, ability to convert daidzein to the metabolite equol, suggests that significant inter-individual variation may be present in the equol-producing capacity of this species, as it is in other species (Setchell 1995; Lampe *et al.* 1998; Lu and Anderson 1998; Setchell *et al.* 2001; Raffi *et al.* 2003; Setchell *et al.* 2003ab). No differences were apparent in the plasma concentration of other daidzein metabolites of this cat, compared to other cats in the study. The production of equol by one cat equates to 16% of the study population. This is lower than the equivalent proportion reported for humans (33%; Setchell and Cole 2006) but the restricted sampling schedule, single dose, and low number of animals included in this study indicate that further studies are necessary before accurate assessment of equol production in cats can be determined. Other metabolites of genistein, which have been detected in humans consuming isoflavones (Joannou *et al.* 1995), were not detected.

Interestingly, genistein monosulphate appeared in its maximum concentration at a time which closely resembled the C_{max} estimated for conjugated (as distinct from unbound) genistein in domestic cats by Cave *et al.* (2007a). Given the observation of this current study that no other forms of conjugated genistein were detectable in feline plasma, it is reasonable to suggest that the conjugated genistein reported by Cave *et al.* (2007a) may have been comprised solely, or at least predominantly, of genistein monosulphate. Previous studies in other species have failed to distinguish between conjugated and

unbound forms of isoflavone in the plasma despite an understanding that the majority of plasma isoflavones occurs in the conjugated form. The potentially gross overestimation of genistein's bioavailability resulting from such studies cannot be understated, especially if its presence in a conjugated form reduces its ability to bind to the oestrogen receptors and may therefore render it less available for activity at cellular concentrations (Barnes *et al.* 2000; Kinjo *et al.* 2004).

Unbound daidzein achieved peak plasma concentration at an equivalent time to unbound genistein, although the sulphate conjugates of daidzein appeared approximately 1.5 h after genistein monosulphate. This may indicate the preferential or enhanced efficiency of sulphation processes towards genistein, compared to daidzein, which may be delayed in its release to circulation. Glucuronidation of genistein has been reported to occur in a more rapid fashion than that for daidzein (Chen *et al.* 2005c) and it is possible that the same occurs for sulphation.

The absolute values reported here should be interpreted with caution since mixing of the tablet may not have been complete and variation in oesophageal transit of the tablets may have resulted in the isoflavones entering the stomach or intestines prior to the bulk of the meal. This may have produced differing absorption and metabolism responses to those observed in cats consuming isoflavones as part of a diet. However, given the very small SEM of the T_{max} for unbound forms variable oesophageal transit is unlikely to have had an effect.

In summary, the identification of daidzein mono- and di-sulphates, and genistein monosulphate in the plasma of domestic cats is reported here for the first time. The potential for conversion of daidzein to equol has also been demonstrated in this species, albeit in only one individual. Furthermore, metabolic capacity for isoflavones by domestic cats appears to be efficient, with only minimal proportions of the ingested amount detected in their unbound forms. This has important implications for the

potential for isoflavones to exert physiological activity in the domestic cat, when consumed at concentrations representative of dietary intake. The findings of this study challenge previous hypotheses of increased susceptibility to isoflavone influence in felids due to a deficient capacity for de-toxification via metabolic conjugation (Setchell *et al.* 1987a; Court and Freeman 2002).

3.2. Experiment Two:

Genistein and Daidzein Metabolism in Domestic Cats Following Ingestion of a Commercially Prepared Feline Diet Known to Contain Isoflavones

3.2.1. Aim

Isoflavones are not naturally consumed by felids as soy extract tablets, but rather as components inherent in the soy ingredients included in a dietary matrix. To this end, the literature suggests that the dietary matrix may modulate isoflavone absorption, compared to purified compounds, or those administered in the absence of food (see Section 1.4.1 and 1.6.2). The aim of this experiment was therefore to determine metabolite production in domestic cats consuming isoflavones as part of a commercially available feline diet.

3.2.2. Materials and Methods

3.2.2.1. *Animals*

Four adult male domestic cats with a mean age of 4.92 years (± 0.72) and BW of 4.56 kg (± 0.21) were included in the trial. The cats were bred and maintained at the Centre for Feline Nutrition, Massey University and the study was approved by the Massey University Animal Ethic Committee (2005). Antibiotics had not been administered to any of these cats for 3 months prior to the trial to prevent disturbance to their normal gut microflora.

3.2.2.1.a. *Pre-trial conditioning/testing*

To ensure gastrointestinal function was not altered by an acute change in diet, and also to confirm diet acceptance, the cats were gradually adapted to the study diet over a 10 day period prior to the onset of the trial. Cats consumed the test diet as their sole source of nutrition for 5 days prior to the onset of the trial. As such, cats were also exposed to isoflavones during this period. In order to minimise the potential error introduced due to overlapping plasma, urinary and faecal excretion of isoflavones from previous meals prior to the study meal cats were fasted for 24 h prior to the onset of the trial. Previous experiments suggested that near-baseline plasma concentrations of isoflavones would be achieved within this time frame (see Appendix 3).

All other pre-trial conditioning and testing was performed as described in Section 3.1.2.1.a.

3.2.2.1.b. *Trial conditions*

For the duration of the trial, cats were individually housed in polythene plastic metabolism cages (0.8 x 0.8 x 1.1 m) equipped with an elevated rest area, feed and water bowl area and litter tray. The cats were exposed to natural light cycles and provided with *ad libitum* access to water.

3.2.2.2. *Isoflavone content of the diet*

A moist, commercially prepared diet was selected from those surveyed in Section 2.1. The selection criteria included local availability, and an isoflavone content representative of the higher end of the range determined in Section 2.1.3.1. The commercial diet selected was a meat and gravy formula, containing 151.44 μg/g DM genistein and 198.43 μg/g DM daidzein, expressed as aglycone equivalents. The forms of genistein or

daidzein were not identified and equol was not screened for due to methodological issues. Following consumption of the study meal, an isoflavone-free base diet was fed for the remainder of the trial. The proximate composition of these diets is described in Table 3.3.

	Dry Matter (DM)	Crude Fat (%)	Crude Protein (%)	Carbohydrate (%)*
Base diet	17.73	25.2	35.6	29.2
Test diet	15.51	23.27	47.01	19.99

Table 3.3. Proximate composition of diets used in experiments in Section 3.2.
*Calculated by difference

3.2.2.3. *Plasma collection*

Placement of indwelling catheters and blood collections was as described in Section 3.1.2.3.a. and 3.1.2.3.b.

3.2.2.4. *Diet administration and sampling schedule*

Following a 24 h fast, a blood sample (T0) was withdrawn from the catheter of each cat to obtain the baseline sample. Each cat was then offered the study diet in an amount calculated to provide 50% of their individual daily ME requirements. Cats were allowed 10 min to consume the meal, and any uneaten food was weighed and recorded. The time that the meal was offered was recorded as T0, and serial blood samples were then withdrawn at 1.5, 2.5, 4, 6, 8, and 12 h after this. The next meal was provided at 12 h and all subsequent meals consisted of an isoflavone-free base diet (also utilised in Section 3.1. and described in Table 3.4). Therefore, no further isoflavones were consumed following the ingestion of the isoflavone-containing meal at time 0. After the final sampling period the catheters were removed. Catheter insertion sites were re-bandaged for a further 24 h to ensure healing, after which time bandages were removed and cats were returned to normal housing conditions.

3.2.2.5. *Plasma analysis*

Plasma samples were analysed as described in Section 3.1.2.5 (LC-MS/MS).

3.2.3. Results

3.2.3.1. *Isoflavone consumption*

Cats consumed an average of 0.62 mg genistein/kg BW (± 0.06 mg/kg BW) and 0.82 mg daidzein/kg BW (± 0.08 mg/kg BW) providing a total mean isoflavone dose of 1.44 mg/kg BW. This is equivalent to an average intake of 9.79 µmol (± 1.13) of genistein and 13.60 µmol (± 1.56) of daidzein, as aglycone equivalents.

At the time of feeding the study meal, three of the four cats consumed less (by an average of 24 g) than predicted from their average intake per meal from the previous five days, whilst one consumed slightly more than anticipated (10 – 25 g).

3.2.3.2. *Plasma metabolites*

One of four cats was found to have detectable concentrations of unbound genistein and daidzein in his plasma. Daidzein monosulphate, genistein monosulphate and equol monosulphate were present in the plasma of all cats. Daidzein disulphate and equol disulphate were detected in three of four cats. The mono- and di-sulphate metabolites of genistein and daidzein (including equol) were present in the baseline (T0) plasma sample of cats. Unbound equol was not detected in any cat's plasma, while dihydrohdaidzein and *O*-DMA were detected in one of the cats. No glucuruonide metabolites were detected in any cat. The T_{max} and C_{max} of isoflavone metabolites are shown in Table 3.5.

One cat (# 10) appeared divergent in his plasma metabolite profile (qualitatively and quantitatively) and as such his plasma data is reported separately (Table 3.4). This cat was the only cat observed to have detectable concentrations of unbound genistein and daidzein, and had simultaneously lower than average concentrations of the monosulphate conjugates of these isoflavones. Likewise, this cat did not produce any disulphate metabolite, whereas the other three cats did. Equol monosulphate was detected in lower concentrations (0.16 μmol/L at only one time point) than observed in other cats (mean 1.43 μmol/L ± 0.71). This cat produced detectable concentrations of dihydrodaidzein and *O*-DMA where the other three cats did not.

Metabolite		T_{max} (h)	C_{max} (μmol/L)
Genistein monosulphate	Mean (n = 3)	1.83 (0.29)	1.78 (0.33)
	Cat # 10	1.5	0.19
Daidzein monosulphate	Mean (n = 3)	1.83 (0.29)	1.33 (0.28)
	Cat # 10	1.5	0.05
Daidzein disulphate	Mean (n = 3)	2.50 (0)	1.46 (0.16)
	Cat # 10	ND	ND
Equol monosulphate	Mean (n = 3)	7.00 (1.15)	1.08 (0.62)
	Cat # 10	ND	ND
Equol disulphate	Mean (n = 3)	8.25 (3.75)	0.64 (0.20)
	Cat # 10	ND	ND
Unbound genistein	Mean (n = 3)	ND	ND
	Cat # 10	1.5	0.26
Unbound daidzein	Mean (n = 3)	ND	ND
	Cat # 10	4.0	0.19

Table 3.4. Mean (± SEM) plasma pharmacokinetic variables for cats (n = 3 + 1) fed a diet containing 151.44 μg/g dry matter (DM) genistein and 198.43 μg/g DM daidzein. Average intake of isoflavones was 0.62 mg genistein/kg body weight (BW) and 0.82 mg daidzein/kg BW. ND = Not detected

The proportion of genistein detected to occur as a conjugate did not vary significantly according to sampling time, ranging from 99.61% (± 0.00) at 1.5 h, up to 100.00% (± 0.00) at 6 and 12 h post-dose. The proportion of daidzein detected in a conjugated form

also did not vary significantly, ranging from 99.61% (± 0.00) at 1.5 h post-dose, to 100.00% (± 0.00) at 4, 6, and 12 h post-dose.

3.2.4. Discussion

Until now the ability of domestic cats to absorb isoflavones has been limited to studies including only dietary supplements or tablets (Section 3.1.3; Cave *et al.* 2007a). The form of isoflavones found in commercially prepared diets may vary considerably to the purified forms found in such supplements and ingestion of these diets may therefore result in divergent kinetics and absorption potential.

This study has now confirmed the ability of dietary isoflavones to cross the intestinal barrier of the cat when ingested as a naturally-occurring part of a feline diet. Furthermore, ingestion of a commercially-prepared diet yielded slightly different metabolite profiles compared to those observed following soy extract administration. In general, the efficiency of isoflavone conjugation was nearly identical to that seen in Section 3.1. On the other hand, the production of equol appeared to be greater in cats consuming isoflavones as part of a dietary matrix compared to cats exposed to soy extract tablets. Additionally, certain other daidzein metabolites (dihydrodaidzein and *O*-DMA) were detected in one of four cats in this study where no cats in the previous study were found to produce these metabolites.

Equol is known to possess greater affinity for oestrogen receptor binding compared to daidzein in other species (Sathyamoorthy and Wang 1997; Branham *et al.* 2002; Lehmann *et al.* 2005; Hwang *et al.* 2006). Equol may therefore subsequently exert greater physiological influence, so the finding that all (4) cats exposed to a daidzein-containing diet were capable of producing equol may have ramifications for the long-term reproductive physiology of household cats.

Whilst the production of equol is thought to be limited to one third of the human population (Setchell *et al.* 2003a; Setchell and Cole 2006), and was only observed in one cat, at one time point, in the study using soy extract (Section 3.1.3), all cats in the present study produced some form of equol. A crucial role for intestinal microflora has been demonstrated for the production of equol and *O*-DMA (Atkinson *et al.* 2005; Cassidy 2005) and equol is thought to be produced by the enzyme activity of microflora in the distal intestine, most likely the colon (Setchell *et al.* 2003a). As such, this metabolite is not known to appear in the plasma of other species until 6 - 8 h post-dose (King and Bursill 1998; Setchell *et al.* 2003a), with peak plasma concentrations typically occurring after approximately 24 h (Setchell 1995; King *et al.* 1996; King 1998; King and Bursill 1998; Setchell *et al.* 2001; Setchell *et al.* 2003a). Hence, if equol production occurs in the domestic cat in a similar manner as other species, the sampling regime of Section 3.1 (i.e. including a final plasma sample at 12 h post-dose) should have been sufficient. However, only one cat exhibited detectable concentrations of equol within this 12 h sampling regime following soy extract exposure, but was detected within 12 h in the current study. This suggests some form of modulatory role for dietary matrix and/or duration of daidzein exposure in the daidzein metabolising capacity of domestic cats.

It has been suggested that chronic exposure to daidzein may increase equol-producing capacity in the gut microflora of mammals (Hedlund *et al.* 2005). Hence, it is feasible that equol production was increased in the current study due to the adaptation period prior to sampling in which they consumed the isoflavone-diet. This is supported by the finding that equol was detectable in the baseline sample of the majority of cats, and overlap from previous meals may explain the earlier peak plasma concentrations observed here, compared to studies in humans in which equol T_{max} was typically reported to occur at 24 h (Setchell 1995; King *et al.* 1996; King 1998; King and Bursill 1998; Setchell *et al.* 2001; Setchell *et al.* 2003a). It is also feasible that equol may have been present in the diet as a result of animal protein ingredients. However, equol is not typically reported to occur in natural food sources and it is more likely to have been detected here as a result of gut microflora metabolism.

The background diet may also have been influential. The isoflavone-free base diet used in Section 3.1. did not include any vegetable matter on its ingredients list, whereas the diet used in this present study was categorised in Section 2.1. as a "soy-containing" diet. The greater vegetable content of the diet consumed by cats in this study may have resulted in an intestinal environment which was more conducive to fermentation and biotransformation of isoflavones *in vivo* (Uehara *et al.* 2001; De Boever *et al.* 2000; Rafii *et al.* 2003; Clavel *et al.* 2005; Decroos *et al.* 2005; Setchell and Cole 2006). Hence this may explain the greater incidence of metabolites such as dihydrodaidzein and forms of equol in the present study, compared to that produced by cats consuming a meat-only basal diet in conjugation with soy tablets.

It could be suggested that inclusion within a dietary matrix permitted equol production through a delaying or protective effect, rendering a greater fraction of the daidzein dose available for bioconversion in the colon. However, this appears unlikely since the majority of metabolites appeared in maximum concentrations at earlier time points following dietary consumption, compared to soy extract exposure (Section 3.1.3). Although effort was made to provide the soy extract in combination with a meal in order to mimic normal dietary intake in Section 3.1, it is feasible that the time taken for dissolution of the tablet within the gastrointestinal tract may have been responsible for the delayed T_{max}.

Additionally, processing effects have also been shown to affect the absorption kinetics of isoflavones in other species (Slavin *et al.* 1998; Allred *et al.* 2005) and may have follow-on effects for their metabolism. Heat treatment involved in the canning process is known to result in transformation of isoflavone forms (Kurzer and Xu 1997; Coward *et al.* 1998; Dixon and Ferraria 2002) and may reduce isoflavones to forms that are more efficiently absorbed (Ismail and Hayes 2005). Hence the isoflavones provided in the canned diet used here may have been comprised primarily of aglycones, whilst it could be predicted that the soy extract tablet provided isoflavones in their glycosidic form. The prerequisite for hydrolysis of glycosides prior to absorption has been reported to delay their

absorption compared to administration of aglycone forms in other species (King *et al.* 1996; Sfakianos *et al.* 1997; Cimino *et al.* 1999; Andlauer *et al.* 2000b; Izumi *et al.* 2000; Setchell *et al.* 2001; Cassidy *et al.* 2006; Kano *et al.* 2006; Steensma *et al.* 2006) and may have effectively delayed absorption of the soy extract-derived isoflavones in Section 3.1. This delay may have resulted in greater degradation of daidzein prior to reaching the colon, such that equol production was lowered in the soy-extract study (Section 3.1) while increasing proportions of administered daidzein aglycone, relative to its glycoside, may have modulated equol production in this current study (Kano *et al.* 2006). However, the confounding effect of a positive isoflavone concentration in baseline samples indicates that further kinetic testing is required to determine the effect of dietary matrix.

Interestingly, one cat failed to produce detectable concentrations of daidzein disulphate or equol disulphate, but this cat was also the only cat observed to have circulating concentrations of unbound daidzein. Moreover, this cat was unique in its production of dihydrodaidzein and *O*-DMA, suggesting that daidzein metabolism differed significantly in this individual compared to other cats.

The production of equol has previously been negatively correlated with the proportion of excreted daidzein (Setchell 1995; Slavin *et al.* 1998; Vergne *et al.* 2007). Likewise, since dihydrodaidzein is an intermediate in the formation of equol from daidzein (Joannou *et al.* 1995; Cimino *et al.* 1999; Rafii *et al.* 2003), it could be assumed that their respective concentrations would also be inversely proportional. The finding that neither dihydrodaidzein nor unbound daidzein were detected in the plasma of the three other cats producing equol disulphates is in agreement with these assumptions. However, the detection of all three metabolic endpoints of daidzein metabolism in one individual indicates that daidzein metabolism did not occur in an all or nothing fashion in this cat. A reduced activity of phase II metabolising processes may be indicated in this individual, and may be associated with the ability to produce dihydrodaidzein and *O*-DMA. Protection of the daidzein molecule against further metabolism to dihydrodaidzein and *O*-DMA may be conferred by the presence of a sulphate moiety. If sulphation was

impaired, a greater fraction of daidzein may be available for bioconversion, either in the liver, other tissues, or following re-secretion into the gut lumen. This may, hence, explain the altered metabolite profile of this individual. This cat was recorded to have ingested the largest quantity of food on the day of the trial and also had the highest intake per kg BW. This higher intake may account for the different metabolites produced and indicates a degree of saturation in the phase II metabolising process.

The degree of sulphate conjugation of genistein, daidzein and equol shown here has important consequences for the predicted biological activity of isoflavones in the domestic cat. Unlike the study of Cave *et al.* (2007a) where approximately 4% of the absorbed fraction at C_{max} was present in an unbound form, cats in this study were found to have less than 1% of their plasma genistein present in an unbound state during peak plasma concentrations. However, Cave *et al.* (2007a) did not identify the metabolites present in the plasma of cats, but instead reported "total" and "free". Therefore, comparison can only be made under the assumption that genistein monosulphate was the only conjugated form of genistein present, as was observed in this study. On the basis of this assumption, it appears likely that conjugation was under some form of rate-limiting process at the dose utilised by Cave *et al.* (2007a) (100 mg/kg BW), as will be discussed in more detail in Section 3.3.4.

3.3. Experiment Three:

Mass Balance Study of Purified Genistein and Daidzein Following Administration to Domestic Cats as a Single Oral Bolus

3.3.1. Aim

The plasma appearance of genistein and daidzein following ingestion has been confirmed in the domestic cat (Sections 3.1.3. and 3.2.3.). The present study aimed to determine the fraction of genistein and daidzein dose recovered in the urine, plasma and faeces of domestic cats following administration of a dose of aglycone isoflavones, at doses representative of typical intake via a commercially prepared diet (see Section 2.1.). A further objective of this study was to describe the maximum concentration, time to maximum concentration and approximate area under the curve for metabolites present in the plasma of cats. By identifying all known metabolites in plasma effort was made to estimate the apparent fraction of dose that was absorbed.

3.3.2. Materials and Methods

3.3.2.1. *Animals*

Eight (4 female and 4 male) domestic short-haired cats, with a mean age of 4.9 years (± 0.46) and average BW of 4.10 kg (± 0.33) were included in the trial. The cats were bred and housed at the Massey University Centre for Feline Nutrition, and had been maintained on a commercially prepared diet of undetectable isoflavone content for 2 weeks prior to initiation of the trial. No antibiotics had been used in any of the animals

for the 3 months prior to the trial to prevent disturbance to their normal gut microflora. The trial was approved by the Massey University Animal Ethics Committee (2006).

3.3.2.1.a. *Pre-trial conditioning/testing*

As described in Section 3.1.2.1.a.

An indigestible marker was added to the cats food for use as a marker of faeces associated with a known ingested meal. Chromic oxide was added in sufficient quantities to each cat's diet in order to colour the food green/blue but without reducing palatability (tested in cats which were not assigned to these experiments). However, subsequent faecal collection following ingestion of this marker yielded no visible discolouration associated with the marker and chemical analysis of the marker was not considered to be economical given the limited value of the marker concentration.

3.3.2.1.b. *Trial Conditions*

As described in Section 3.1.2.1.b.

3.3.2.2. *Isoflavone dose*

Genistein and daidzein were administered in their aglycone forms as purified (99% pure) powders (LC Labs, MA, USA). Each isoflavone was weighed out (to within 3 dp) to provide 1 mg genistein/kg BW and 1 mg daidzein/kg BW, and were enclosed in a gelatin capsule (kindly donated by Dr. K. Markwick-Rutherfurd) prior to administration to each cat. An average of 15.25 μmol (± 1.19) genistein and 16.14 μmol (± 1.28) daidzein was delivered to each cat.

The selection of the isoflavone dose employed was based on the results of dietary analyses of domestic cat and cheetah diets (see Section 2.1.3. and 2.2.3.). The total

isoflavone intake of adult cheetahs was estimated to range from 0.00 to 1.47 mg/kg BW, while the median isoflavone intake of domestic cats consuming moist diets ranged 0 – 5.48 mg/kg BW) so a total dose of 2 mg/kg BW was estimated to represent approximate exposure in both species. The dose employed in Section 3.1 was higher than utilised here due to its inclusion in a pre-prepared commercially available tablet. This study aimed to provide the most comprehensive data, applicable to cats consuming isoflavone-containing diets and it was therefore necessary to utilise a more dietarily relevant dose.

3.3.2.3. *Isoflavone administration and sampling schedule*

The isoflavones, genistein and daidzein, were administered in a single capsule at a known time. Serial blood samples were collected thereafter. The capsules were given to cats following intake of base diet (Table 3.3 in Section 3.2.2.2) and food consumption was permitted for a further 5 min following isoflavone capsule administration.

Cats were fasted for 18 h prior to administration of the capsule to ensure maximal consumption of the meal. The capsule was placed at the back of the throat of each cat, 5 min following the offering of a meal, and throat massage was applied until the capsule was swallowed. The time of consuming the capsule was recorded and is herein referred to as time 0. Thereafter, the schedule of blood collection was 0.5, 1.5, 2.5, 4, 6, 8, 12, 24, 48 and 96 h post-isoflavone administration. After the final sampling period the catheters were aseptically removed. Catheter insertion sites were re-bandaged for a further 24 h to ensure healing, after which time bandages were removed and cats were returned to normal housing conditions.

3.3.2.4. *Urine collection*

Urine was collected by means of a purposefully-designed, two-tier litter tray system. A top tray of 1 mm stainless-steel wire mesh was placed inside a solid plastic litter tray that

allowed for the collection of uncontaminated urine. A complete urine sample was collected from each cat during the 24 h prior to the administration of the isoflavone tablet. For the duration of the trial (96 h) all urine voided was collected and pooled into 24 h batches, mixed by stirring and then weighed before being stored at -4°C, prior to analysis. Isoflavones are known to be stable in urine stored at room temperature for 14 days (Atkinson *et al.* 2005) such that it was not necessary to collect urine at intervals shorter than this.

3.3.2.5. *Faecal collection*

Faeces were collected from the top wire tray of each cat's litter tray (described in Section 3.3.2.4.) for 120 h. Faeces were collected in 24 h pooled samples, and then accurately weighed and frozen at -4°C, prior to analysis.

3.3.2.6. *Plasma collection*

3.3.2.6.a. *Catheter placement*

Catheter placement and blood collection technique was as described in Section 3.1.2.3.a. The only exception was that anaesthesia was induced with tiletamine and zolazepam (Zolatil 100; 12 mg/kg BW, sub-cutaneous) instead of propofol, since these cats did not tolerate the intra-venous injection required for propofol.

3.3.2.6.b. *Blood collection technique*

As described in Section 3.1.2.3.b.

3.3.2.7. *Urine analysis*

Urine was analysed for total and unbound genistein and daidzein by High Performance Liquid Chromatography (HPLC) using methodology adapted from Cave *et al.* (2007a). All samples were analysed in duplicate. Conjugated isoflavones were quantified after enzymatic hydrolysis to yield total unbound equivalents. This hydrolysis step required 0.8 ml urine to be combined with 0.8 ml acetate buffer (pH 5), containing 1990 U sulphatase/ml and 205 U glucuronidase/ml (Sigma-Aldrich, St. Louis, MO, USA). Samples were then incubated in a shaking water bath at 37°C for 1 h before being evaporated to dryness in an automatic speed-vac concentrator with a vapour net (Savant, New York, NY, USA). The enzyme incubation phase was eliminated when analysing unbound isoflavone content, and the conjugated proportion estimated by the subtraction of the unbound content from the total equivalent content.

Three methanol extractions were performed on each sample by the addition of 300 µl methanol (BDH, Poole, UK). Suspensions were then mixed by sonication and vortex, before centrifugation at 13,400 rpm for 5 min. The supernatant was taken to dryness before being re-suspended in 200 µl aqueous methanol (4.94mol/l) and the mixing and centrifugation procedure repeated. The supernatant was then withdrawn, and 25 µl injected onto the HPLC system.

Analysis was conducted on an Alliance Waters 486 (Millipore Corp., Milford, MA., USA) HPLC, using a Luna 5 µ C18 reverse-phase column (4 x 250 mm; Phenomenex, Torrance CA, USA), with an in-line 4 x 3 mm C18 guard column (Phenomenex, Torrance CA, USA). Samples were injected in 95 % buffer A (1.75 mol/L HPLC-grade acetic acid (BDH, Poole, UK)) and 5 % buffer B (19.2 mol/L HPLC-grade acetonitrile (BDH, Poole, UK)) and eluted using a linear gradient of 5 % B to 70 % B over 40 min with a 15 min equilibration at 70 % B before returning to starting conditions. The flow rate was set at 0.5 ml/min and isoflavones were detected by absorbance at 260 nm using a Waters W486 UV Detector (Millipore Corp., MA., USA). Quantification was performed using

Millennium software package (Waters Millipore Corp., Milford, MA, USA) based on peak area.

3.3.2.8. *Faecal analysis*

Faeces were accurately weighed, before being freeze-dried and re-weighed. Samples were homogenised using a food processor, further mixed by shaking and then pooled as follows; faeces voided in the period between time 0 and time 48 h were analysed as one sample, as were faeces voided between 48 and 96 h, with a final (third) pooled sample including all faeces voided between 96 and 120 h post-isoflavone ingestion.

Approximately 2 g of each pooled sample was weighed (to 2 dp) into a 50 ml centrifuge tube. Ten ml of 80% methanol (Burdick & Jackson, Michigan, USA) in water was added before being vortexed for 1 min. The sample was then centrifuged and an aliquot removed and filtered through a 0.45 μ syringe filter (BD Pore, Belgium). One ml was taken up for analysis and 10 μl of acetic acid added to prevent peak splitting over time (observed in preliminary experiments). An aliquot of 20 μl was injected onto the HPLC column (Shimadzu Corporation, Kyoto, Japan). Pre-treatment of samples with hydrochloric acid (10 ml of 6 M HCl in methanol, 1:9) was performed on selected samples for extraction of total genistein, daidzein and equol.

A reverse phase C_{18} HPLC column (100 x 4.6 mm, 5 μ) (Applied Biosystems, CA, USA) fitted with a guard column (15 x 3.2 mm, 7 μ) (Applied Biosystems, CA, USA) was used. The HPLC conditions involved a gradient elution with mobile phase A consisting of 40% methanol/water and mobile phase B consisting of 70% methanol/water. Over the first 10 min the gradient was ramped to 100% A, followed by a linear gradient to 100% over the subsequent 10 min. This was held for 9 min before being ramped back to 100% A, over the following 1 min. The total run time was 40 min with a flow rate of 1.5 ml/min. Ultra violet (diode array) detection (Shimadzu Corporation, Kyoto, Japan) was utilised with

wavelength set at 260 nm for daidzein and genistein, and 280 nm for equol. Genistein and daidzein standards were supplied by Acros Organics (Geel, Belgium) and reported as 99% and 98% pure, respectively. Equol standard (99% purity) was supplied by Fluka (Sigma-Aldrich, Switzerland). Retention times for daidzein, genistein and equol were 13.2 min, 17.6 min and 14.1 min, respectively.

3.3.2.9. *Plasma analysis*

Analysed as per methodology described in Section 3.1.2.5. (LC-ESI-MS/MS).

3.3.2.10. *Pharmacokinetic modelling*

All values are presented on a molar basis, unless otherwise specified.

The plasma pharmacokinetics of genistein, daidzein, and their respective metabolites were characterised with non-compartmental modelling by WinNonLin Professional (version 4.1., Pharsight Corp, NC, USA). Examination of the plasma-time concentration curves of isoflavone metabolites indicated that elimination of isoflavones followed first order kinetics (with a relatively constant fraction eliminated per unit time). Linear regression of the terminal log-linear phase of the concentration-time curve was used to determine the elimination rate constants. The linear trapezoidal rule, with extrapolation to infinity, was applied to estimate the area under the curve ($AUC_{0-\infty}$). Other parameters determined were peak plasma isoflavone concentration (C_{max}) and time required to achieve the peak concentrations (T_{max}).

The amount recovered in any compartment (plasma, urine or faeces) or a combination thereof, was calculated according to the following formula:

$$\text{Recovery (\% of dose)} = \frac{\text{Amount detected over the study duration}}{\text{Ingested dose}} \times 100$$

The $AUC_{0-\infty}$ was used to estimate the amount detected in the plasma compartment. The fraction of absorbed dose excreted in the urine was estimated as:

$$\text{Urinary excretion (\% of absorbed fraction)} = \frac{\text{Total recovered in the urine}}{AUC_{0-\infty}} \times 100$$

Conjugates detected in the urine and faeces were analysed after hydrolysis and therefore expressed as unbound equivalents. This data was therefore included on a molar basis, after multiplying the mass amount by the molar weight of either genistein or daidzein. However, plasma conjugates were expressed as μmol of each specific conjugated molecule (e.g. disulphate or monosulphate). Therefore, the molar amount of conjugated metabolites were normalised to give equivalent parent compound (genistein or daidzein) amounts.

3.3.2.11. *Statistical analyses*

Residual data was tested for normality with the Anderson-Darling test. Where data were normally distributed, differences were tested for significance using a one-way ANOVA. Where data were not normally distributed, comparisons were performed using a Kruskal-Wallis test with the significance concentration set at 0.05. All statistical analyses were performed using MINITAB (version 15, Minitab Inc., PA, USA).

Individual pharmacokinetic parameters were calculated for each cat and values then averaged. Unless otherwise stated, all data are presented as mean (± SEM). Where no difference was detectable between genders, the mean for all animals is reported.

3.3.3. Results

3.3.3.1. *Urinary excretion*

No isoflavones were detected in urine samples beyond 96 h and therefore data for samples collected beyond 96 h is not reported. A representative chromatogram from the HPLC assay of feline urine is presented in Figure 3.2. Mean fractional urinary recovery of genistein was 4.87% (± 0.67), which was significantly higher than the fractional urinary recovery of daidzein (1.50% ± 0.29; $p < 0.05$). As a fraction of the amount absorbed (estimated from the $AUC_{0-\infty}$), 9.16% (± 0.90) of the absorbed genistein was excreted in the urine, which was not significantly different from the fraction of absorbed daidzein excreted in the urine (8.65% ± 3.22).

	Genistein		Daidzein	
	Males	Females	Males	Females
Fraction of ingested dose excreted in urine (0 – 72 h) (%)	3.90 (0.48)^{c}	5.84 (1.12)^{c}	1.04 (0.18)^{a}	2.15 (0.36)^{b}
Fraction of plasma content excreted in urine (0 – 72 h) (%)	10.7 (1.19)^{a}	7.60 (0.88)^{a}	12.0 (5.88)^{a}	5.34 (2.53)^{a}
Conjugation extent (% of total excreted detected as a conjugate) in urine	93.6 (1.63)^{a}	91.8 (1.60)^{a}	84.5 (0.71)^{a}	82.2 (5.02)^{a}

Table 3.5. Urinary excretion (mean (± SEM)) of isoflavones by cats following a single oral bolus dose of purified aglycones of genistein and daidzein (providing 1 mg/kg BW each). N = 4 female, 4 male. Different superscripts within rows indicate significant differences ($p < 0.05$).

Both genistein and daidzein appeared primarily as conjugates (Table 3.5). The fraction of ingested daidzein excreted in the urine was significantly greater in females than males ($p < 0.05$), but no difference was detected when calculated as a fraction of the absorbed amount. No difference in the fractional recovery of genistein was detectable between

genders (Table 3.5). No effect of gender was detectable in the proportion of excreted fraction occurring as a conjugate for either isoflavone.

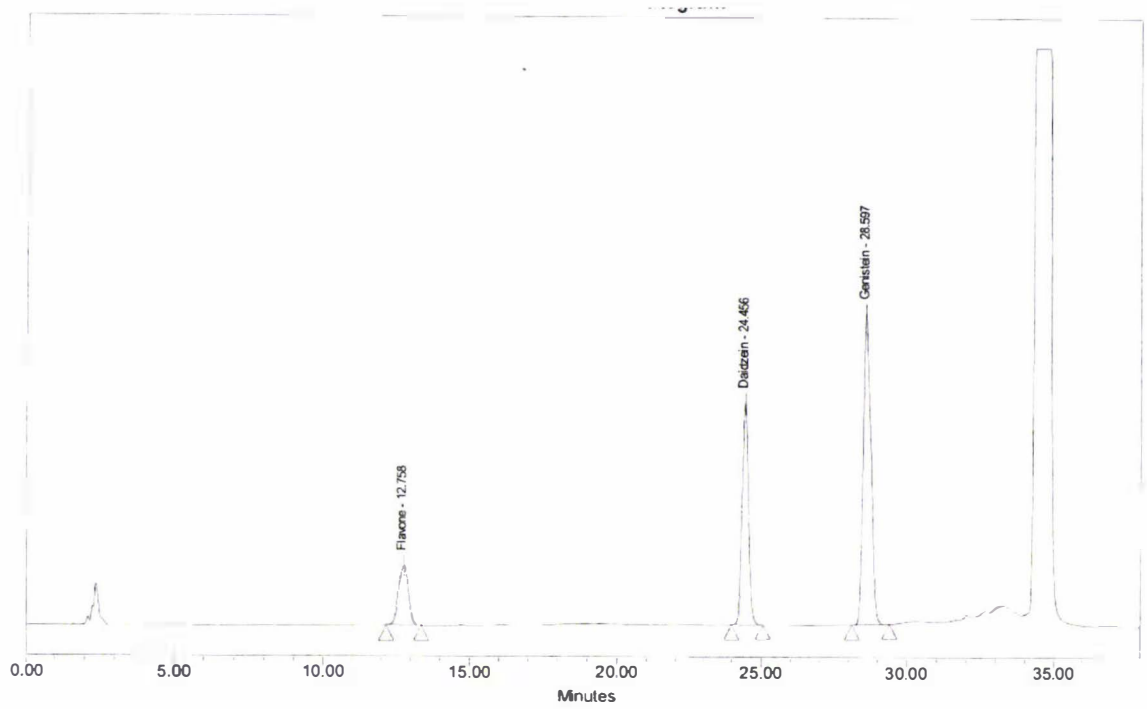


Figure 3.2. Representative HPLC chromatogram from urinary analysis demonstrating the elution of flavone (internal standard), daidzein and genistein.

Females excreted an average total of 0.67 μmol (± 0.10) conjugated genistein and 0.22 μmol (± 0.03) conjugated daidzein. Males excreted a mean of 0.64 μmol (± 0.06) conjugated genistein and 0.16 μmol (± 0.03) daidzein. Unbound genistein and daidzein was excreted in significantly smaller concentrations, with a mean of 0.06 μmol (± 0.02) unbound genistein and 0.05 μmol (± 0.02) unbound daidzein excreted by females. Males excreted an average of 0.04 μmol (± 0.01) unbound genistein and 0.02 μmol (± 0.01) unbound daidzein. Cumulative urinary excretion is illustrated in Figure 3.3.

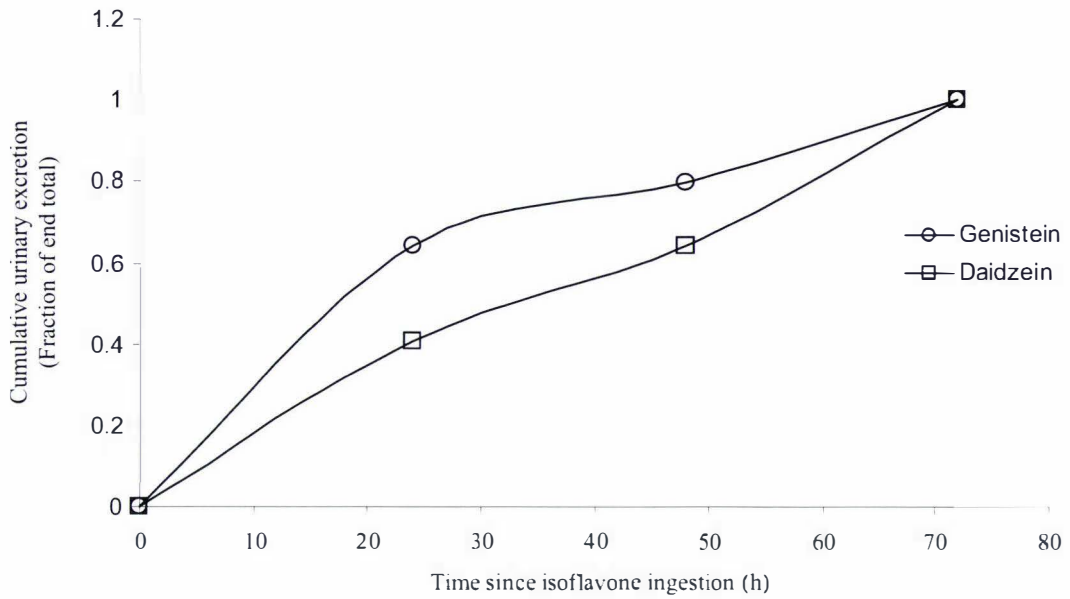


Figure 3.3. Cumulative urinary excretion isoflavones by cats (male and female) following a single oral bolus dose of purified genistein and daidzein, each provided at 1 mg/kg BW.

3.3.3.2. *Faecal excretion*

No samples were found to contain detectable isoflavone concentrations beyond 120 hours and data for this period is therefore not reported. Conjugation of genistein was calculated as the proportion of total genistein excreted in the faeces that occurred as a conjugate (overall mean 10.19% ± 4.05). The proportion of conjugated genistein detected `was greater than that of daidzein (overall mean 0.56% ± 0.59; $p < 0.05$). No significant difference was detectable between the proportion of ingested dose of genistein and daidzein appearing in the faeces in conjugated or unbound form ($p > 0.05$). However, the fraction of ingested genistein recovered in the faeces of males was significantly higher than that of females ($p < 0.05$) (Table 3.6). A trend towards greater faecal recovery of ingested daidzein dose was also detected for males, compared to females, although this was not statistically significant ($p < 0.1$). Cumulative faecal excretion is depicted in Figure 3.4.

	Genistein		Daidzein	
	Males	Females	Males	Females
Fraction of dose excreted (%)	35.8 (4.10)[a]	32.3 (4.54)[bc]	39.6 (6.57)[ac]	34.6 (4.35)[ac]
Fraction of dose excreted as conjugates (%)	6.00 (3.30)	1.90 (1.06)	0.27 (0.27)	0
Conjugation extent (% of total excreted)	15.7 (6.98)	5.52 (2.50)	1.18 (1.18)	0

Table 3.6. Mean faecal excretion (± SEM) of isoflavones by domestic cats following a single oral bolus dose of 1 mg genistein/kg BW and 1 mg daidzein/kg BW. N = 4 males, and 4 females. Different superscripts within rows indicate significant differences ($p < 0.05$).

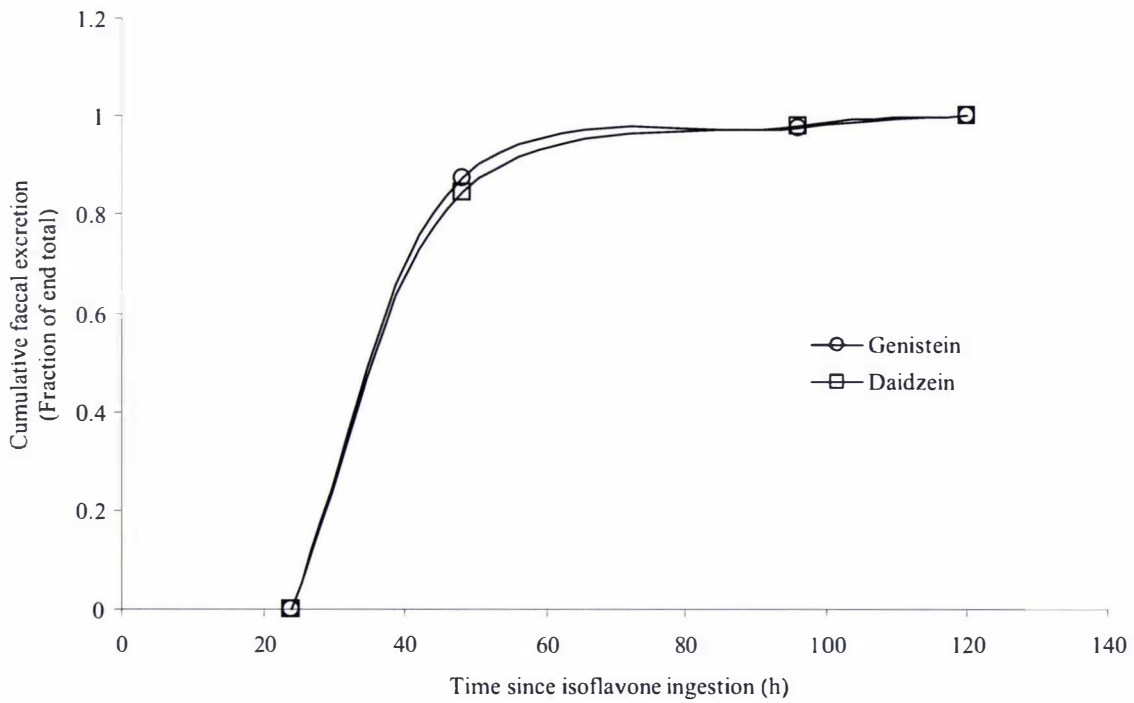


Figure 3.4. Cumulative faecal excretion of unbound isoflavones by cats (male and female) following a single oral bolus dose of purified aglycone isoflavones, each provided as 1 mg/kg BW.

3.3.3.3. *Plasma pharmacokinetics*

Five (1 female, 4 males) of eight cats (62.5%) were found to have detectable concentrations of unbound daidzein in their plasma, whilst only two of eight cats (25%) had detectable concentrations of unbound genistein in their plasma. Neither unbound isoflavone were detected in plasma at all time points for each cat. Therefore, $AUC_{0-\infty}$ of unbound forms could not be estimated and conjugation of plasma metabolites is reported as 100%. Daidzein monosulphate, daidzein disulphate and genistein monosulphate were detected in all cats. No cat excreted unbound equol, dihydrodaidzein or *O*-DMA or any glucuronide conjugate at any time point. Only two cats excreted equol monosulphate and one cat excreted equol disulphate. Table 3.7 describes pharmacokinetic variables of the detected metabolites for which sufficient data existed to enable pharmacokinetic analysis.

Metabolite	T_{max} (h)	C_{max} (µmol/L)	AUC 0 - ∞ (h*µmol)
Genistein monosulphate	4.25 (0.77)	2.20 (0.26)	10.43 (0.96)
Daidzein monosulphate	6.88 (0.99)	0.22 (0.02)	2.06 (0.43)
Daidzein disulphate	6.25 (0.88)	0.31 (0.04)	6.36 (1.18)

Table 3.7. Plasma pharmacokinetic (mean ± SEM) variables for cats (n = 8) following a single oral bolus of purified genistein and daidzein (1 mg genistein/kg BW and 1 mg daidzein/kg BW). T_{max} = time of maximum peak plasma concentration, C_{max} = peak plasma concentration, AUC = area under the curve.

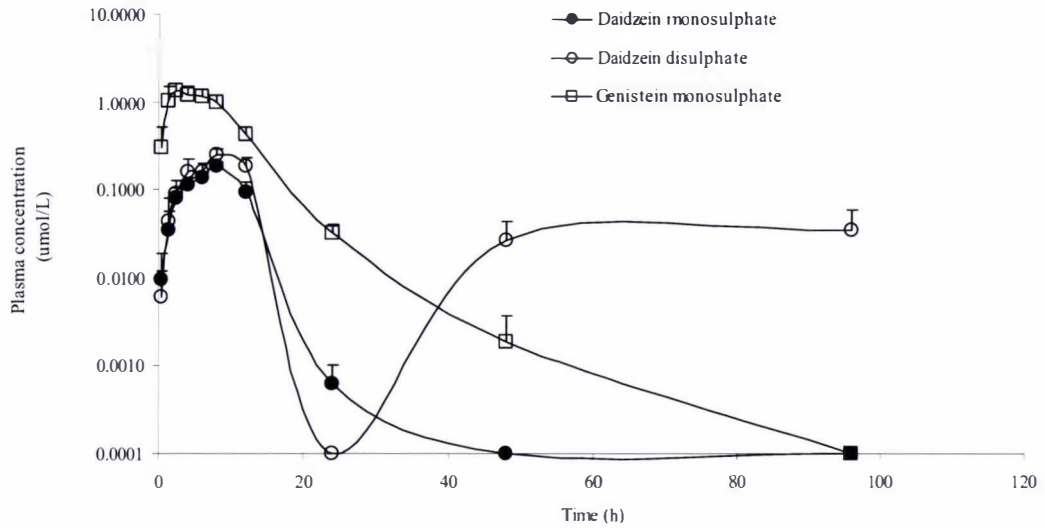


Figure 3.5. Log/linear plot of the plasma concentration of genistein and daidzein metabolites (normalised to parent compound molar amounts) in cats following ingestion of 15.25 µmol genistein aglycone and 16.14 µmol daidzein aglycone. Graph depicts mean concentration. The error bars depict SEM (note: they are one-sided due to a limitation in the graphical software).

In cats with detectable concentrations of unbound daidzein, the C_{max} of daidzein monosulphate, as well as that of daidzein disulphate, were both significantly greater than that of unbound daidzein ($p < 0.05$). Insufficient data points were available for statistical comparison of the C_{max} of unbound genistein and genistein monosulphate.

Maximum plasma concentration for unbound genistein was significantly greater than that of unbound daidzein ($p < 0.05$). Genistein monosulphate C_{max} was significantly greater than that of daidzein monosulphate ($p < 0.05$). No difference was detectable between genders in pharmacokinetic parameters ($p > 0.05$). Plasma concentration-time curves for the sulphate conjugates are illustrated in Figure 3.5.

Detectable concentrations of unbound genistein were found in circulation in only two cats (1 male, 1 female) and only at the sampling point 24 h post-dose. The concentration at

this time was 0.37 μmol/L and 0.61 μmol/L in the two cats. In cats with detectable concentrations of unbound daidzein, this metabolite appeared at only one or two time points. The time of unbound daidzein appearance in the plasma was 0.5 h (0.01 μmol/L) in one cat and 48 h (0.24 μmol/L) in a second cat.

Equol monosulphate was detected in two cats at either 8 (0.02 μmol/L) or 12 h post-dose (0.04 μmol/L). Equol disulphate was only detected at one time point (4 h post-dose), in one cat, at a concentration of 0.09 μmol/L.

3.3.3.4. *Fraction Appearing in the Plasma and Mass Balance Recovery*

The fractions of ingested dose appearing in the plasma, urine and faeces are described in Table 3.8. The mean total genistein fraction voided from the body (i.e. accounted for in urine and faeces combined) was 38.89% (± 2.97), which was similar to the voiding of daidzein which was 38.77% (± 3.63). Plasma recovery in combination with urine and faeces was not calculated due to the potential to count the same isoflavone twice.

As a fraction of the absorbed amount (estimated from the $AUC_{0-\infty}$), 8.47% (± 0.79) of the absorbed fraction of genistein appeared in the urine as conjugates and 0.69% (± 0.15) as unbound metabolites. These values did not differ significantly from those of daidzein (7.62% ± 3.17 appearing in the urine as conjugates, and 1.03% ± 0.23 appearing in the urine in the unbound form).

Total recovery was calculated by summing amounts detected in plasma and faeces. Total mean recovery of genistein was 87.94% (± 7.71) of ingested dose, which was significantly greater than that of daidzein (66.4% ± 6.96, $p < 0.05$).

	Genistein		Daidzein	
	Conjugated	Unbound	Conjugated	Unbound
Urinary recovery	4.47% (0.53)	0.36% (0.09)	1.25% (0.20)	0.25% (0.08)
Faecal recovery	3.95% (1.78)	30.1% (2.35)	0.13% (0.13)	37.1% (3.77)
Plasma recovery	53.9% (3.24)	0	29.2% (6.30)	0

Table 3.8. Fractional recovery (of ingested dose) of genistein and daidzein in male (n = 4) and female (n = 4) domestic cats.

3.3.4. Discussion

Exposure to purified aglycone forms of genistein and daidzein resulted in the efficient sulphation of absorbed isoflavones, which was also observed when cats consumed soy extract and dietary sources of isoflavones. Conjugates are also reported to be the principal forms of isoflavones reported to occur in the plasma of other species (Sfakianos *et al.* 1997; Setchell *et al.* 2001; Coldham *et al.* 2002b; Allred *et al.* 2005), although these conjugates are primarily reported to be glucuronides (Sfakianos *et al.* 1997; Andlauer *et al.* 2000a) indicating different metabolic processes are involved in the conjugation of isoflavones in cats. Yet, regardless of mechanism and despite previous hypotheses to the contrary, domestic cats appear to be more efficient in the conjugation of dietary isoflavones than some species. Furthermore, the limited activity of UDP-glucuronosyltransferases in the liver of the cat (Hietanen and Vaino 1973; Schmoldt *et al.* 1982; Court and Greenblatt 1997 and 2000) may be inconsequential for the metabolism of isoflavones in the cat.

Indeed, unbound genistein accounted for up to 11% of the total detected genistein in rats at T_{max} (Coldham *et al.* 2002b), and 10% in dogs (McClain *et al.* 2005), whereas complete conjugation of the dose was observed in the majority of cats studied here (Section 3.1.3, 3.2.3 and the present study). Although both rats and dogs were exposed to much higher doses of genistein such that saturation of conjugating pathways was therefore feasible, even at doses as high as 100 mg/kg BW (Cave *et al.* 2007a), conjugated genistein still accounted for 96% of the plasma genistein content at T_{max}. This

suggests that some interspecific variation may exist in the conjugation capacity of cats, rats and dogs, and that dietary isoflavones may have divergent, potentially (comparatively) low physiological activity in felids.

Differences in metabolite production were observed in cats consuming varying forms of isoflavones. The drop in plasma daidzein disulphate concentration and its subsequent re-appearance (Figure 3.5) is unexplained and may represent some form of re-cycling or an error in analysis. In contrast to previous findings following dietary exposure (Section 3.2.3), equol monosulphate and disulphate were not consistently detected in cats in this study. As discussed previously (Section 3.2.4), the single bolus dose provided in this study may have been insufficient to produce equol in all potential equol producers. Alternatively a source of dietary equol may have been present in the diet used in Section 3.2, which was unavailable in the base diet used here. This appears unlikely given the rarity with which equol is reported in dietary sources. Dihydrodaidzein and *O*-DMA do not appear to be common metabolites in the cat, regardless of the duration of exposure since no cat in the present study, and only one cat in the previous study (Section 3.2.3), exhibited detectable concentrations of these metabolites. This may be due to the efficient sulphation observed, or may reflect interspecific differences in gut microflora.

Although efficient sulphation of absorbed isoflavones has been determined in the cat, the enzymes responsible for phase II metabolism in other species are known to exhibit a saturation threshold (Sfakianos *et al.* 1997; Murato *et al.* 2002). Comparison of the findings of this and previous studies (Section 3.1 and 3.2) with the literature (Cave *et al.* 2007a) indicate that saturation of phase II metabolism pathways may occur at higher doses since the proportion of absorbed fraction present as a conjugate was reported to be lower in a study using a dose 100 times greater than used here (Cave *et al.* 2007a). Alternatively, absorption may be reduced in a dose-dependent manner, which is supported by the observation that a greater fraction of ingested dose was recovered in the plasma of cats in the present study, compared to the reported bioavailability of conjugated genistein in cats exposed to 100 mg/kg BW (Cave *et al.* 2007a).

Yet despite this, only a minor fraction appeared unbound at high doses (Cave *et al.* 2007a), indicating any saturation or absorption threshold is unlikely to be reached following intake of concentrations achievable from typical dietary sources. This suggests that the cat's intestinal and/or hepatic capacity for genistein conjugation is efficient and highlights the fact that without the differentiation between metabolite forms, the reporting of total isoflavone equivalents would have led to the gross overestimation of the genistein and daidzein availability in these situations. Given that the different metabolic forms of isoflavones are thought to confer vastly divergent physiological influence (Barnes *et al.* 2000), the consequences for inaccurately predicting biological activity are striking. In reality, this type of phase II metabolism is likely to reduce the oestrogenic potency and receptor binding ability of isoflavones (Kinjo *et al.* 2004). As such, the majority of the absorbed dose may be rendered biologically inert. Therefore, this study further demonstrates that previous assumptions regarding the apparently deficient phase II metabolic capacity of the cat (Setchell *et al.* 1987ab; Court and Freeman 2002), may be fundamentally misleading.

Consistent with other species (Xu *et al.* 1994; Lu *et al.* 1995; Xu *et al.* 1995; King *et al.* 1996; Setchell *et al.* 2003b), genistein occurred in greater concentrations than daidzein in the plasma of cats, which may reflect greater enteric transport of genistein compared to daidzein (Chen *et al.* 2005b). However, exposure to isoflavones in their aglycone form produced divergent pharmacokinetic and metabolite profiles to those observed in cats following dietary exposure. Compared to the findings of Section 3.2.3, the time taken for genistein and daidzein sulphates to achieve maximum plasma concentration following a dose of purified aglycone forms was delayed, but was similar to that observed for soy extract tablet administration (Section 3.1.3). It is likely that this is an artefact of the tablet or capsule used to provide the isoflavone dose in Section 3.1 and the current study. The apparently increased rate of absorption of isoflavones when occurring as part of a feline diet may reduce intestinal biodegradation or transformation, the consequence of which

may include a greater fraction of ingested dose being recovered and/or enhanced equol production.

Urinary excretion of genistein and daidzein showed an approximately logarithmic decay in the mass excreted over time. The lower urinary excretion of genistein in males, compared to females, is consistent with the higher faecal excretion in males, potentially indicating a reduced absorption in males, or increased enterohepatic recirculation and subsequent biliary excretion. Of note is the finding that only approximately 10% of either absorbed genistein or daidzein was recovered in the urine. This is strongly suggestive of alternative excretory routes (e.g. hepatic), metabolism or degradation to unidentified forms, and/or extensive tissue storage.

Interestingly though, the fraction of genistein excreted in the urine was greater than that of daidzein. This is in contrast to previous findings in other species (Xu *et al.* 1994; King and Bursill 1998; Lu and Anderson 1998; Shelnutt *et al.* 2000; Setchell *et al.* 2003a), in which daidzein is reported to have enhanced urinary excretion over genistein. The reason for the divergent urinary profile in the present study is unclear but may be associated with the efficient sulphation of both isoflavones as the process of sulphation is likely to improve isoflavone solubility in the urine (Sfakianos *et al.* 1997). Indeed a lower relative proportion of daidzein was found in its conjugated form in the urine and this may have contributed to its overall lower urinary excretion. Likewise, primarily glucuronides have been shown to be involved in enterohepatic recirculation of genistein in other species, and the large proportion of genistein sequestered in the bile is thought to contribute to its lower urinary excretion (Sfakianos *et al.* 1997). Hence, the lack of glucuronide metabolites of genistein in the cat may infer a reduced biliary uptake, leaving a greater fraction of genistein available for urinary excretion. In this situation, the plasma ratio of genistein to daidzein would be more likely to mirror the urinary ratio, which is not normally the case in other species (Watanabe *et al.* 1998; Shelnutt *et al.* 2000; Bloedon *et al.* 2002; Shelnutt *et al.* 2002; Setchell *et al.* 2003b; Zhang *et al.* 2003). Alternatively, a

more substantial fraction of the daidzein dose may be degraded, or metabolised to unidentified forms in the cat, thereby reducing the fraction accounted for in the urine.

Faecal excretion of both genistein and daidzein appeared to occur in a more distinct manner than urinary excretion and the percentage of isoflavone dose recovered in the urine was higher than that reported in humans (0.4 – 4.4%; Xu *et al.* 1994 and 1995; King 1998; Watanabe *et al.* 1998). However it was similar to the 35% of ingested genistein dose reported in rats (King *et al.* 1998; Steensma *et al.* 2006), in which rapid entero-hepatic recirculation is known to occur, with over 60% of the dose recovered in the bile (Sfakianos *et al.* 1997). Therefore, the fraction of genistein dose recovered in the faeces of cats here may include a re-circulated fraction. Alternatively, since it is likely that enterocytes are responsible for first-pass metabolism of isoflavones in the cat as they are in other species, they may represent important sinks of isoflavones. If enterocytes were sloughed prior to release of the isoflavone metabolites, these cells may also contribute to the faecal isoflavone conjugate content.

Support for entero-hepatic recirculation of genistein is provided by the observation that the proportion of faecal genistein occurring in a conjugated form (which was not administered orally) was higher than that of daidzein. Yet, the plasma appearance of genistein was not biphasic, and the fraction of faecal genistein recovered in a conjugated form was minimal (< 16% in males or < 6% in females), such that entero-hepatic recirculation, if present, is unlikely to represent a major pathway in the cat. Hence, the fraction of unbound isoflavones detected in the faeces may represent the fraction that was transported through the gastrointestinal tract without modification or absorption. Alternatively, deconjugation of biliary conjugates may also have occurred during intestinal transit or conjugated forms may represent enterocyte re-secretion rather than biliary secretions. Future studies are warranted to establish the extent of enterohepatic recirculation of isoflavones in cats.

Calculation of isoflavone recovery included only plasma $AUC_{0-\infty}$ and faecal excretion. Urinary excretion was excluded from this calculation since it is likely that isoflavones measured in the plasma would be duplicated in the urine at some time point. However, it must be noted that both faecal and plasma concentrations may also include a proportion of absorbed and re-secreted isoflavones. Hence the values may overestimate total recovery and predicted absorption extent. Approximately 12% of the genistein dose was not accounted for in this study. Moreover, the average fraction of genistein voided from the body within 96 h of dosing (i.e. urinary and faecal excretion) was less than 40% of the ingested dose. It is possible that a large proportion of the dose was retained in an enterohepatic recirculation, or alternatively stored in tissues. Accumulation of genistein in the liver and reproductive tissues has been reported previously and the circulating plasma concentration is therefore likely to underestimate tissue exposure (Chang *et al.* 2000; Tsai 2005; McClain *et al.* 2006b). Furthermore, the tissue stores are reported to contain a greater proportion of unbound genistein than conjugated genistein than predicted from plasma concentrations (McClain *et al.* 2005; McClain *et al.* 2006b).

A larger fraction of ingested daidzein dose was missing from the recovered amount in the plasma and faecal compartments, whereby approximately one third of the dose was unaccounted for. As observed for genistein, the mean fraction of daidzein voided from the body within the study period was less than 40%. Equol and other daidzein metabolites were not consistently detected in these cats and, hence, cannot explain the low recovery rate. This may indicate significant tissue accumulation of daidzein. Alternatively, the structure of daidzein may confer it with a greater chance of escaping biodegradation or absorption prior to reaching the colon, thereby permitting its further metabolism to unidentified forms.

Of particular note was the finding that daidzein disulphate approached baseline levels by 24 h but then reappeared later in significant plasma concentrations. This would support colonic absorption of daidzein and subsequent sulphate conjugation but may otherwise indicate significant release from tissues and/or enterohepatic recirculation. Moreover, the

finding that this metabolite had not neared baseline levels at 96 h post-dose suggests the terminal elimination phase is likely to be extended compared to other metabolites. The greater potential for tissue accumulation and prolonged systemic exposure of this metabolite may confer greater biological activity. Further investigation of the pharmacokinetics of daidzein, with increased terminal elimination phase sampling points is warranted to confirm this since assay inaccuracy at very low concentrations may have been a factor.

Interestingly, Coldham and Sauer (2000) recovered 100% of the administered genistein dose in rats when cage washings and animal carcasses were included in the calculation. Deposition of genistein into animal carcasses accounted for less than 1% of the administered dose, while cage washings included up to 3 % of the dose. The relatively high proportion of dose recovered in cage washings may reflect incomplete collection of urine and faeces, or remnants of the dose that were shed in the hair or skin of the animals. However, the study of Coldham and Sauer (2000) utilised a standard rodent diet, which is likely to have included unreported concentrations of genistein (Brown and Setchell 2001) that may have erroneously contributed to the fraction recovered in cage washings. Nonetheless, it is possible that minute amounts of urine and/or faeces were not collected from metabolism cages in the present study. Likewise, the concentration of isoflavones in the shed hair and skin of cats was not measured and could account for some losses.

In order to assess true bioavailability an intravenous dose would need to be administered for comparison and a greater number of sampling points would be necessary in the terminal elimination phase. However, in the absence of these, the fraction of genistein dose absorbed (as calculated from the plasma AUC) by cats in this study (54%) represents a crude estimate of its availability for biological activity. Bioavailability of conjugated genistein has previously been investigated in cats consuming 100 mg/kg BW, and estimated to be 30%, but that of unbound genistein was only 1.3% (Cave *et al.* 2007a). Therefore, the findings of the present study indicate that although a greater fraction of genistein may be absorbed when ingested in a lower dose, conjugation is more

efficient at lower doses. Since the unbound forms are thought to possess greater affinity for tissue sequestration and receptor binding, the true availability of genistein in this study may be reduced due to the lack of unbound forms in circulation. However, significant accumulation of unbound genistein may occur in the body tissues (Chang *et al.* 2000), and therefore the potential exists that the tissue concentration of unbound forms may greatly exceed that of the plasma, in these cats. Consequently the biological availability and potential activity of isoflavones may be significantly underestimated. However, the availability of isoflavones after a single bolus dose is likely to differ to that estimated at steady state. Furthermore, it is not known whether AUC or C_{max} is more important in predicting potential biological activity. Further research is warranted.

3.4. Conclusions

- The cat is able to absorb, excrete and metabolise the dietary isoflavones, genistein and daidzein. The predominant form of isoflavone metabolites present in the plasma of domestic cats were sulphate conjugates, although low concentrations of unbound parent compounds, equol, dihydrodaidzein, and *O*-DMA were also detected in some cats.

- The plasma content of genistein and daidzein represented 54% and 29% of ingested dose, respectively. This is higher than the bioavailability of genistein in cats administered with 100 mg/kg BW genistein, possibly due to the existence of a saturation threshold in conjugation capacity at high doses. However, true bioavailability was not calculated in this study.

- Faecal elimination is the principal pathway of excretion and it remains to be determined if the fraction voided in the faeces underwent enterohepatic recirculation or passed through the gastrointestinal tract without absorption. Poor absorption is likely to be an important cause of the low plasma recovery in this species. Urinary excretion represented a minor proportion of the absorbed fraction of both genistein and daidzein, indicating alternative excretory mechanisms, degradation to unidentified forms, or tissue accumulation.

- The efficient conjugation of genistein and daidzein exhibited by the domestic cat indicates the potential for biological perturbation by isoflavones may be reduced in this species, compared to rodents and humans. However, tissue concentrations were not measured and may represent significant pools of unbound forms.

- A single oral bolus of daidzein does not result in the consistent appearance of equol in the plasma of domestic cats, although equol was detected in a limited number of cats, but only at one or two points in time. However, prior exposure to dietary daidzein was associated with the consistent appearance of equol metabolites in the plasma of domestic cats.

- Ingestion as part of a dietary matrix, compared to soy extract or purified forms appears to affect absorption kinetics, as well as equol production. Likewise, significant dose-related effects may be apparent. Further testing is required.

- Gender-specific differences were observed although the small sample sizes utilised indicate the need for further investigation before these can be confirmed. Overall, isoflavone availability and metabolism are not considered to differ dramatically between genders.

CHAPTER FOUR:

The Absorption, Excretion and Metabolism of Genistein and Daidzein in Captive Cheetahs (*Acinonyx jubatus*)

4.0. Introduction

Dietary isoflavones have been found in diets fed to captive cheetahs (Section 2.2.3) and may be absorbed by cheetahs, as they are in cats (see Section 3.1 to 3.4). However, the cheetah may differ in its capacity to absorb and metabolise isoflavones. Hence, determining the capacity of cheetahs to absorb, metabolise and excrete dietary isoflavones was important for two reasons. Firstly, by determining the absorption and metabolism of isoflavones in the cheetah, it may be possible to predict the potential for biological activity that these compounds are capable of exerting in this species. Secondly, it was necessary to determine the suitability of the domestic cat as a model for the influence of isoflavones in the reproductive and hepatic health of cheetahs (as in Section 5 and 6). Experiments were designed to describe plasma concentration-time curve, and metabolite profile, as well as urinary and faecal excretion of genistein and daidzein in cheetahs, following consumption of an oral bolus of soy isoflavones.

4.1. Absorption and Disposition Kinetics, of Genistein and Daidzein in Captive Cheetahs (*Acinonyx jubatus*)

4.1.1. Aim

The aim of this experiment was to establish the absorption and disposition kinetics of genistein and daidzein and their metabolites in the cheetah. The analysis of the plasma, urinary and faecal metabolites provides descriptive information regarding isoflavone metabolism in the cheetah. For evaluation of potential age-related differences in conjugation capacity, urinary isoflavone metabolites were assayed in juvenile cheetahs.

4.1.2. Materials and Methods

4.1.2.1. Animals

4.1.2.1.a. *Cubs*

Five healthy, male cubs used in this study, ranging from 15 – 30 days of age at the time of urine collection. Cubs were bred at De Wildt Cheetah and Wildlife Centre, South Africa, and hand-reared by the author at Cheetah Outreach, South Africa. Cubs were fed a commercially available milk replacer (Kitty Milk®, Kyron Laboratories, South Africa) which included soy protein in its ingredient list. The amount consumed per feed was recorded and equated to an average intake of 6.06 mg/kg BW genistein and 1.95 mg/kg BW daidzein per day during the collection period. No antibiotics had been used in these cubs prior to obtaining the urine samples.

4.1.2.1.b. *Adults*

Four adult male cheetahs were included in the study and housed by either Wellington Zoo, New Zealand (n = 2), or Cincinnati Zoo and Botanical Gardens, USA (n = 2). These cheetahs were bred at De Wildt Cheetah and Wildlife Centre, South Africa and hand-reared by the author, prior to transfer to the USA or NZ. All adult cheetahs were of good health, with a mean body weight of 45.08 kg (± 2.08), and mean age of 3.33 years (± 1.32). Ethical approval for the conduct of this study was granted by the individual facilities involved. Antibiotics had not been administered to the cheetahs for 3 months prior to the onset of the trial.

The diet of the adult cheetahs in New Zealand consisted of animal muscle meat and whole carcasses (chicken, turkey, goat, guinea pig and horse), supplemented with vitamin and mineral powders. The diet used by the American facility was a commercially prepared horse meat-based diet. This diet was analysed previously in Section 2.2, and found to be devoid of detectable concentrations of either daidzein or genistein.

4.1.2.2. *Isoflavone dose*

The diet of cheetah cubs was determined (according to methods described in Section 2.1.2.) to contain 48.85 μg/g dry matter (DM) daidzein and 151.4 μg/g DM genistein.

As in Section 3.1.2, isoflavones were administered to adult cheetahs as a soy extract tablet (Phytolife1®: One a Day, Healthy Living, Palmerston North), containing isoflavone glycosides from 26.8 g dry seed *Glycine max* (soy bean). Significant time intervals were experienced between sampling at each facility and since isoflavone tablets had a limited shelf-life, it was not possible to use the same batch of tablets for all the cheetahs.

Each batch of isoflavone tablets were analysed individually. The cheetahs in the USA were administered tablets analysed to contain 15.11 mg genistein and 15.83 mg daidzein each, whilst the cheetahs assessed in New Zealand were given tablets containing 16.76 mg genistein and 18.37 mg daidzein each. A total of four tablets were administered to each cheetah, providing either 60.44 mg or 73.48 mg genistein and 63.32 mg or 67.04 mg daidzein, depending on the batch.

The cheetahs utilised in the plasma pharmacokinetic evaluation (New Zealand facility) were provided a dose of 2.28 mg genistein aglycone and 64.76 mg conjugated genistein. The daidzein component of their dose comprised 4.08 mg daidzein aglycone and 73.48 mg conjugated daidzein. The mean dose of genistein was 1.40 mg/kg BW (± 0.08) and daidzein 1.49 mg/kg BW (± 0.09), or a total isoflavone dose of 2.89 mg/kg BW (± 0.16).

4.1.2.3. *Isoflavone administration*

Isoflavones were administered to juvenile cheetahs in six discrete meals on a daily basis. Adult cheetahs were administered with isoflavones as a single bolus of soy extract tablets, which were hidden in a piece of meat and fed concurrently with a meat-only meal.

4.1.2.4. *Urine Collection*

Complete collection of all urine voided by cubs was not possible due to an inability to isolate cubs. Voided samples (1 per cub) were collected into plastic specimen vials following ano-genital stimulation to elicit urination, after bottle feeding.

Adult cheetahs (n = 4) were housed on a non-porous surface for the duration of the trial, in order to facilitate total urine collection. Urine was collected after natural voiding, by syringe-suction recovery from the floor surface, before transfer into collection vials. A

baseline urine sample was obtained from each cheetah prior to administration of the tablets, and collection continued for a total of five days beyond administration of the tablets. The time and weight of each voiding was recorded. Urine was stored at -20°C prior to analysis for isoflavone content.

4.1.2.5. *Faecal collection*

All faeces voided by adult cheetahs (n = 4) for five days following administration of the tablets were collected into plastic bags. Faeces were stored at -20°C, prior to freeze-drying and analysis for isoflavone content. The time and weight of each voiding was recorded.

4.1.2.6. *Plasma collection*

Multiple plasma samples could not be obtained in the cheetahs due to an inability to place indwelling catheters. Hence, critical time points in the plasma concentration-time curve of the domestic cat (see Section 3.3) were selected for use here. Although the plasma time points are not suited to the prediction of bioavailability, the use of an extended sampling regime and the mass balance design are thought to permit a reasonable estimate of the fraction absorbed.

Plasma was collected from adult cheetahs (n = 2) at one facility (Wellington Zoo, New Zealand). Blood (2 ml per sample) was collected from conscious cheetahs by venipuncture of the saphenous vein and transferred into heparinised vacutainers. Within 15 min of collection samples were then centrifuged for 10 min at 3000 rpm, and the plasma collected into labelled tubes. Plasma was stored at -20°C until analysed. Blood was collected thereafter at 2, 4, 6, 10, 24 and 56 h post ingestion of tablets. One cheetah did not permit the voluntary withdrawal of blood at the final time point so data from this animal is included up to 24 h post isoflavone administration. More frequent sampling

was not possible without sedation or placement of indwelling catheters in the cheetahs. These factors were not feasible in cheetahs due to the risks of anaesthesia, the endangered status of these animals and their public display as part of the zoo's collection.

4.1.2.7. *Urine analysis*

Urine samples were pooled into 24 h collections and analysed for both total and unbound genistein and daidzein, by High Performance Liquid Chromatography (HPLC) according to methodology described in Section 3.3.2.7.

4.1.2.8. *Faecal analysis*

Faeces were pooled into three periods, namely faeces voided within the first 24 h of the onset of the trial, faeces voided between 24 and 72 h and a third period of faeces voided between 72 and 96 h following administration of isoflavone tablets. Faeces were analysed for unbound genistein, daidzein and equol, as well as total genistein and daidzein, according to methodology described in Section 3.3.2.8.

4.1.2.9. *Plasma analysis*

In order to identify the individual metabolites, a liquid chromatography (LC)-electrospray (ESI)-mass spectrometry(MS)/MS assay was used to measure genistein, daidzein and their metabolites in cheetah plasma, according to methodology described in Section 3.1.2.5.

4.1.2.10. *Pharmacokinetic modelling*

All values are expressed on a molar basis, unless otherwise specified.

The plasma pharmacokinetics of genistein, daidzein, and their respective metabolites were characterised with non-compartmental modelling by WinNonLin Professional (version 4.1, Pharsight Corp, NC, USA). Examination of the plasma-time concentration curves of isoflavone metabolites indicated that elimination of isoflavones followed first order kinetics (with a relatively constant fraction eliminated per unit time). Linear regression of the terminal log-linear phase of the concentration-time curve was used to determine the elimination rate constants. The linear trapezoidal rule, with extrapolation to infinity, was applied to estimate the area under the curve ($AUC_{0-\infty}$). Other parameters determined were peak plasma isoflavone concentration (C_{max}) and time required to achieve the peak concentrations (T_{max}).

The amount recovered in any compartment (plasma, urine or faeces) or a combination thereof, was calculated according to the following formula:

$$\text{Recovery (\% of dose)} = \frac{\text{Amount detected over the study duration}}{\text{Ingested dose}} \times 100$$

The $AUC_{0-\infty}$ was used to estimate the amount detected in the plasma compartment. The fraction of absorbed dose excreted in the urine was estimated as:

$$\text{Urinary excretion (\% of absorbed fraction)} = \frac{\text{Total recovered in the urine}}{AUC_{0-\infty}} \times 100$$

Conjugates detected in the urine and faeces were analysed after hydrolysis and therefore expressed as unbound equivalents. This data was therefore included on a molar basis, after multiplying the mass amount by the molar weight of either genistein or daidzein. However, plasma conjugates were expressed as μmol of each specific conjugated molecule (e.g. disulphate or monosulphate). Therefore, the molar amount of conjugated

metabolites were normalised to give equivalent parent compound (genistein or daidzein) amounts.

4.1.2.11. *Statistical analyses*

Residual data was tested for normality with the Anderson-Darling test and found not to be normally distributed. Comparison between genistein and daidzein was performed using a Kruskal-Wallis test with the significance level set at 0.05. All statistical analyses were performed using MINITAB (version 15, Minitab Inc., PA, USA). Individual pharmacokinetic parameters were calculated for each cat and values then averaged. Unless otherwise stated, all data are presented as mean (± SEM).

Individual pharmacokinetic parameters were calculated for each cat and values then averaged. Unless otherwise stated, all data are presented as mean (± SEM).

4.1.3. Results

4.1.3.1. *Urinary excretion*

4.1.3.1.a. *Cubs*

The median genistein concentration in cub urine was 0.006 mg/ml and ranged from 0.002 mg/ml to 0.009 mg/ml. The median daidzein concentration was 0.005 mg/ml and ranged from 0.002 mg/ml up to 0.007 mg/ml. The concentration of total genistein in each sample did not differ significantly from that of total daidzein ($p > 0.05$). Of the recovered genistein, an average of 64.1% (± 1.43) was present as a conjugate of either glucuronic acid or sulphate. The methodology did not permit quantification of individual metabolites. The proportion of recovered daidzein, present in the conjugated form, was 72.6% (± 4.36). The results presented here represent steady state conditions since

samples were collected from animals consuming isoflavones regularly throughout the day.

4.1.3.1.b. *Adults*

Over the 72 h urine collection period an average total of 11.9 μmol (± 5.74) conjugated genistein and 2.24 μmol (± 0.85) unbound genistein was detected in the urine of cheetahs. An average total of 30.26 μmol (± 10.9) conjugated daidzein and 2.68 μmol (± 0.47) unbound daidzein was recovered in the same period. As a fraction of the ingested dose, 5.69% (± 1.98) of genistein and 13.16% (± 4.09) of daidzein was detected in the urine. The metabolites of both genistein and daidzein were primarily glucuronic acid or sulphate in the urine of cheetahs, where 66.1% (± 16.3) of genistein, and 82.7% (± 10.0) of daidzein was conjugated.

No significant difference was detectable between the fraction of the dose of genistein and daidzein recovered in the urine ($p > 0.05$).

4.1.3.1.c. *Age-related conjugation capacity*

The proportion of conjugated genistein was moderately higher in adults compared to cubs, but this was not significant ($p > 0.05$). The proportion of daidzein occurring in the urine in its conjugated form was significantly higher ($p < 0.05$) in adult cheetahs (82.7 ± 10.0%) compared to cubs (72.6 ± 4.36%).

4.1.3.2. *Faecal excretion*

Since cheetahs did not pass faeces on a daily basis, a large amount of variation existed in the daily excretion rate of ingested isoflavones. However, isoflavones were most concentrated in faeces voided between 48 – 96 h. Cheetahs consistently excreted one

voiding with markedly higher concentrations of isoflavones compared to all other voidings in which isoflavones were detectable. There was no significant difference between the fraction of ingested genistein and daidzein dose appearing in the faeces of cheetahs ($p > 0.05$).

	Genistein	Daidzein
Total excreted in 72 h (μmol)	158.0 (31.1)	116.4 (5.44)
Fraction of dose excreted (%)	66.58 (11.88)	45.3 (23.6)
Fraction of dose excreted as conjugates (%)	16.9 (11.9)	0.49 (0.35)
Fraction of dose excreted as unbound parent forms (%)	58.1 (± 5.47)	45.0 (± 2.36)

Table 4.1. Mean faecal excretion of isoflavones by adult cheetahs following a single oral bolus dose of 4 soy extract tablets, providing an average of 1.40 mg/kg BW (± 0.08) and daidzein 1.49 mg/kg BW (± 0.08). N = 4 males.

An average of 137.1 μmol (± 13.8) unbound genistein and 41.8 μmol (± 29.6) conjugated genistein was detected in cheetah faeces. An average of 115.8 μmol (± 5.16) unbound daidzein and 1.34 μmol (± 0.95) conjugated daidzein was detected in cheetah faeces.

4.1.3.3. *Plasma pharmacokinetics*

On a molar basis, cheetahs in the pharmacokinetic study were provided 247.7 μmol genistein and 271.5 μmol daidzein. Genistein occurred as either its unbound form or as genistein monosulphate in the plasma of cheetahs. Daidzein occurred as its unbound form, daidzein monosulphate or daidzein disulphate.

One cheetah had detectable concentrations of unbound equol in its plasma at one time point (2 h post dose) and at a concentration of 0.004 μmol/L in this cheetah. Equol disulphate and monosulphate were not detected. Dihydrodaidzein was detected in one cheetah, at one time point (2 h post dose), with a concentration of 0.001 μmol/L. The

metabolite *O*-DMA was detected consistently in one cheetah (Table 4.2), but not at all in the second cheetah.

Metabolite	T_{max} (h)	C_{max} (µmol/L)	AUC 0 - ∞ (h*µmol/L)
Unbound Genistein	2.0, 2.0	0.55, 0.31	4.15, 3.1
Unbound daidzein	2.0, 2.0	0.35, 0.20	2.30, 3.08
Genistein monosulphate	2.0, 2.0	12.20, 9.40	103.2, 104.0
Daidzein monosulphate	2.0, 2.0	2.25, 2.48	29.6, 33.3
Daidzein disulphate	ND, 2.0	ND, 0.05	ND, 0.90
O-DMA	2.0, ND	0.03, ND	0.64, ND

Table 4.2. Plasma pharmacokinetic (cheetah 1, cheetah 2) variables for cheetahs, following a single oral bolus of soy extract, providing 247.72 µmol genistein and 271.51 µmol daidzein. T_{max} = time of maximum peak plasma concentration, C_{max} = peak plasma concentration, AUC = area under the curve. ND = not detected

Conjugation of genistein in the plasma was 96.1% in one cheetah, and 97.1% in the second cheetah. Daidzein conjugation was lower than genistein; 92.8% in one cheetah, and 91.0% in the second cheetah.

The plasma concentration-time curve of unbound genistein and daidzein in cheetahs is illustrated in Figure 4.1. The plasma-time concentration curves for genistein and daidzein monosulphate in cheetahs, are illustrated in Figures 4.2a and 4.2b, respectively.

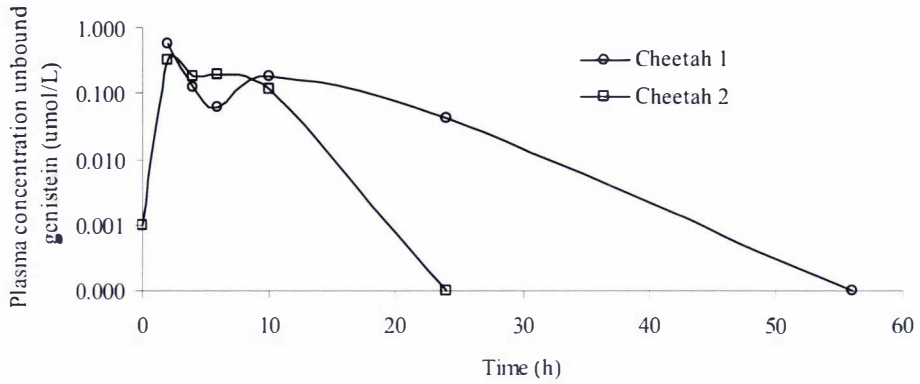


Figure 4.1a. Plasma concentration-time curve of unbound genistein in two cheetahs, following dietary exposure to 247.7 μmol genistein, plotted on a log-linear scale.

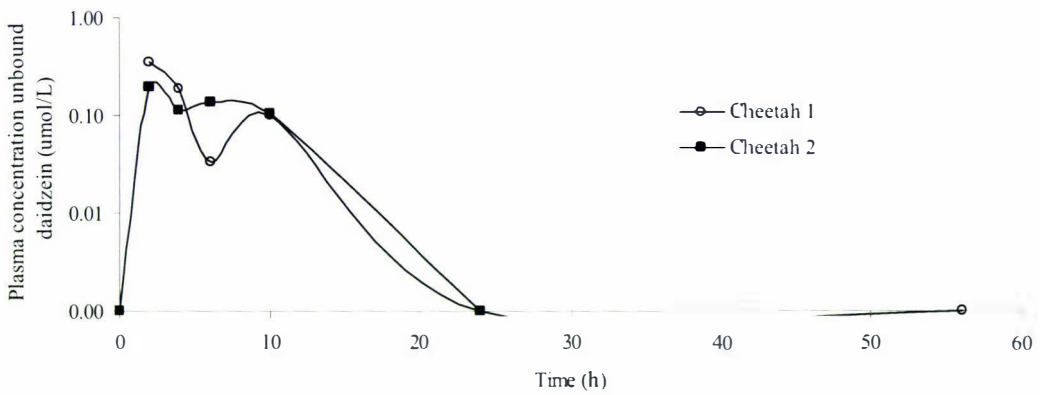


Figure 4.1b. Plasma concentration-time curve of unbound daidzein in two cheetahs, following dietary exposure to 271.5 μmol daidzein, plotted on a log-linear scale.

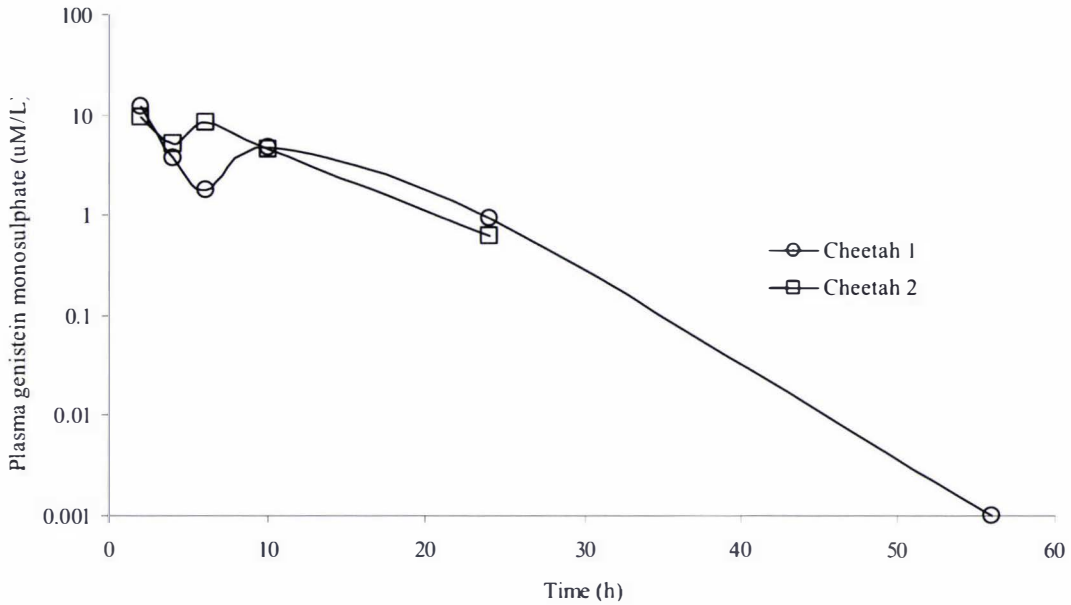


Figure 4.2a. Plasma concentration-time curve of genistein monosulphate in two cheetahs, following dietary exposure to 247.7 μmol genistein, plotted on a log-linear scale.

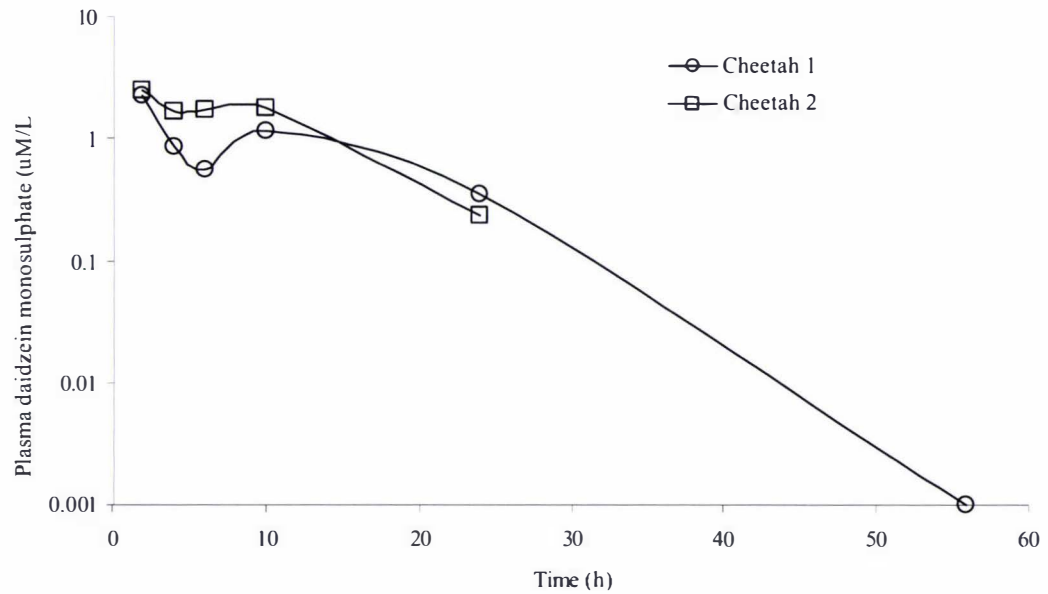


Figure 4.2b. Plasma concentration-time curve of daidzein monosulphate in two cheetahs, following dietary exposure to 271.5 μmol daidzein, plotted on a log-linear scale.

4.1.3.4 *Fraction Appearing in the Plasma and Mass Balance Recovery*

The AUC of genistein monosulphate and unbound genistein estimated a total recovery of 83.6 μmol and 83.1 μmol genistein in two cheetahs, respectively. As a fraction of the ingested dose, this estimated that 33.8% and 33.6% of the genistein dose was absorbed. The AUC of daidzein monsosulphate, disulphate and unbound daidzein estimated a total recovery of 24.8 μmol and 28.4 μmol daidzein in two cheetahs, respectively. As a fraction of the ingested dose, this estimated that 9.13% and 10.5% of the daidzein dose was absorbed.

As a fraction of the amount absorbed (estimated from the plasma content of 2 cheetahs) 14.4% and 33.6% of plasma genistein was excreted in the urine of the two cheetahs, respectively. Greater concentrations of daidzein appeared in the urine of cheetahs, than it did in their plasma. The fraction of absorbed daidzein which was excreted in the urine of two cheetahs represented 135.5% and 202.8% of the absorbed amount.

4.1.4. Discussion

The doses of administered genistein and daidzein used here were approximately equivalent to those predicted to be ingested by a cheetah consuming a commercially-prepared, soy-containing carnivore diet, manufactured for use in zoological facilities (see Section 2.2.3.). Furthermore, these doses were similar to those proposed by Setchell *et al.* (1987ab) to have elicited changes in reproductive tracts and livers of captive cheetahs. Although it would have been preferable to administer isoflavones via a commercial diet to ensure absolute relevance to the captive setting, this was not feasible since the cheetahs were not currently consuming an isoflavone-containing diet, and dietary manipulation was not permitted.

This study expands on the work of Setchell *et al.* (1987) as it demonstrates the ability of cheetahs to absorb, excrete and metabolise the isoflavones genistein and daidzein.

Approximately one third of the dose of genistein appeared in the plasma of cheetahs, while less (approx. 10%) of the ingested daidzein dose was detectable in the plasma. These crude estimates of availability suggest that genistein may be no more or less available for biological activity in the cheetah, than they are in other non-felid species (Coldham *et al* 2002b; Cave *et al* 2007a). However, the fraction of unbound genistein recovered in the cheetah (< 3.5%), was lower than previously reported in the rat (6.8% - 14.6%; Coldham and Sauer 2002b), indicating that the availability of this biologically active form may be reduced in the cheetah.

Unfortunately the availability of daidzein is less well studied in other species but is predicted to be similar, although perhaps slightly lower than that of genistein due to differences reported in its uptake and structural affinity (Murota *et al.* 2002). However, the production of daidzein metabolites (e.g. equol), such as observed in one cheetah, may underestimate its true availability and potential for biological activity. The area under the curve reported for total daidzein equivalents in humans (Setchell *et al.* 2003b) was not dissimilar to that determined here for the daidzein sulphate conjugates. Yet, given the finding that a greater concentration of daidzein was excreted in the urine than detected in the plasma, it is possible that the absorption of daidzein in the cheetah was greater than estimated here. Further testing is warranted.

The conjugation of genistein and daidzein appeared to be efficient, although involved only the addition of one or two sulphate moieties. Unlike non-felid species (Setchell 1995; Sfakianos *et al.* 1997), glucuronides were not detected in the plasma of cheetahs. This supports the notion that the cheetah is similar to the domestic cat in its glucuronidating ability (Court and Greenblatt 2000; Krishnaswamy *et al.* 2004). A supposed inability to metabolise isoflavones has previously been thought to increase the susceptibility of cheetahs to the potential biological effects of isoflavone exposure (Setchell *et al.* 1987ab). However, the findings of this study indicate that the cheetah is capable of efficient phase II metabolism, and utilises sulphation instead of glucuronidation for this purpose. Indeed, the degree of conjugation of circulating forms

of genistein and daidzein was over 90%, which is similar to that reported in the dog (McClain *et al.* 2005) and human (Setchell *et al.* 2001). Therefore, it is unlikely that the adult cheetah will be any more susceptible to physiological perturbation following isoflavone exposure than observed in these species due to differences in metabolism.

However, the conjugation capacity for genistein and daidzein in neonatal cheetahs may be reduced, compared to adults. It is possible that collection at steady state in the cubs, compared to following a single bolus dose in adults could explain the difference, if chronic exposure resulted in a saturation of the conjugating pathways. Likewise, the higher dose per kg body weight provided by the cub's diet may also have resulted in a saturation threshold being exceeded. Due to limitations in the study design and low sample sizes this finding requires further investigation. However, findings in human infants support a reduced conjugating capacity in neonates (Setchell *et al.* 1998; Doerge *et al.* 2002; Rafii *et al.* 2004; Atkinson *et al.* 2005; Cassidy 2005). This indicates that the feeding of isoflavones to cheetah cubs has the potential to expose these animals to greater concentrations of unbound forms of isoflavones than predicted by studies of adult felids. Since the unbound forms are known to possess higher relative binding affinities for the oestrogen receptors (Kinjo *et al.* 2004), caution is warranted in the use of isoflavone-containing milk replacers for cheetah cubs.

Urinary excretion of genistein and daidzein (as a fraction of dose) was similar to that reported for other species (Rowland *et al.* 2003). No difference was detected between the fractional recovery of daidzein and genistein in the urine, although sampling with a greater number of animals may increase the power to detect a difference. Typically a greater fraction of daidzein is reported to be excreted in the urine in other species (Xu *et al.* 1994; King and Bursill 1998; Lu and Anderson 1998; Shelnutt *et al.* 2000; Setchell *et al.* 2003a) and results of this study indicate a trend in this direction.

Of note was the observation that greater concentrations of daidzein appeared in the urine of cheetahs than detected in the plasma. This indicates that the blood sampling regime may have been insufficient to accurately calculate the total amount of daidzein absorbed. Hence, the absorption capacity of cheetahs towards daidzein, and potentially genistein may be significantly greater than estimated here. Alternatively, since the metabolites present in the urine were not identified, it is feasible that some metabolites were not identified and quantified in the plasma but were detected in the urine following enzymatic processing.

Interestingly, the faecal concentration of unbound genistein and daidzein were higher than reported for other species (Xu *et al.* 1994 and 1995; King 1998; Watanabe *et al.* 1998). If the fraction detected in the faeces was not absorbed, or subjected to entero-hepatic recirculation, this finding suggests that the cheetah may be impaired in its absorption capacity for isoflavones, compared to other species. The methodology employed here does not allow elucidation of this. However, the majority of faecal isoflavones were voided in one sample, which is suggestive that these isoflavones travelled through the GIT directly and escaped absorption.

The production of equol was only observed in one cheetah. The small sample size does not allow interpretation as to the proportion of cheetahs likely to possess an equol-producing capacity. Furthermore, the fact that only a single oral bolus dose was provided may have resulted in insufficient exposure to instigate significant production of equol. As indicated in cats (Section 3.3.4), it is possible that equol production may become more apparent in cheetahs following chronic exposure. Of interest is the observation of consistent production of *O*-DMA in one cheetah. This metabolite is a known end product of daidzein metabolism in humans (Joannou *et al.* 1995) and the limited production of its intermediate (dihydrodaidzein) may indicate that the bioconversion of daidzein to equol was efficient or occurred without the production of an intermediate metabolite in this individual.

4.2. Comparison between domestic cats and cheetahs

This section is included to identify and discuss the differences and similarities in isoflavone pharmacokinetics and availability between domestic cats and cheetahs. The findings of this section are relevant to ascertaining the validity of using the domestic cat as a model for the cheetah in regards to the potential for reproductive and/or hepatic perturbation by dietary isoflavones. Comparison of urinary and faecal excretion is made between cats (N = 8) evaluated in Section 3.3, where an equivalent dose of genistein and daidzein was administered, and cheetahs (N = 4) described in Section 4.1.2.1.b. In cases where a sex-specific difference was detected in Section 3.3, only data for male domestic cats (N = 4) was used to compare with male cheetahs. In the comparison of plasma pharmacokinetics, absorption and metabolite profiles, data from 6 male domestic cats (Section 3.1) were compared with data from 2 male cheetahs (Section 4.1.3.2 and 4.1.3.4). In all cases cheetah and domestic cats were of equivalent sex and age and isoflavone dose (per kg BW) was approximately the same. In the comparison of metabolites the form of isoflavone administered was also identical. However, the dose utilised in cheetah and cat data for comparison of plasma pharmacokinetics, fraction absorbed as well as urinary and faecal excretion differed. In these cases domestic cats were provided with a dose of purified isoflavones whilst cheetahs ingested a soy extract tablet.

4.2.1. *Urinary excretion*

A comparison was made between cheetahs ingesting a soy extract tablet and domestic cats administered with purified genistein and daidzein, of similar dose (per kg BW). There were no statistically significant interspecific differences detected between the fractional excretion of genistein ($p > 0.05$) when compared on a equi-dose basis (Table

4.3). However, a trend ($p < 0.10$) towards greater fractional excretion of daidzein by cheetahs was observed.

	Genistein		Daidzein	
	Domestic cats	Cheetahs	Domestic cats	Cheetahs
Fraction of dose excreted (%)	4.87	5.69	1.50	13.2
Proportion conjugated (%)	93.6	66.1	84.5	82.7

Table 4.3. Comparative urinary excretion (Mean ± SEM) of isoflavones when fed to domestic cats as purified forms or cheetahs as a soy extract. N = 8 domestic cats and 4 cheetahs.

4.2.2. *Faecal excretion*

A comparison was made between cheetahs ingesting a soy extract tablet and domestic cats administered with purified genistein and daidzein, of similar dose (per kg BW). The proportion of ingested genistein dose that was recovered in the faeces of cheetahs (66.6%) was significantly higher than that of domestic cats (35.8%; $p < 0.05$). However, no interspecific difference was detectable in the proportion of ingested dose of daidzein appearing in the faeces ($p > 0.05$). The pattern of genistein conjugation being higher than that of daidzein was common to both species. The proportions of genistein and daidzein occurring in cheetah faeces in a conjugated form (16.9% and 0.49%, respectively) were not different to that detected in domestic cat faeces (15.7% and 1.18%, respectively).

4.2.3. *Plasma pharmacokinetics and metabolism*

A comparison was made between cheetahs ingesting a soy extract tablet and domestic cats administered with purified genistein and daidzein, of similar dose (per kg BW). The proportion of genistein conjugates in the plasma of cheetahs (96.1 – 97.1%) was significantly lower than that of domestic cats, which was nearly 100% (Section 3.3; $p < 0.05$). The proportion of daidzein conjugation in cheetah plasma (91.0 – 92.8%) was

significantly lower than that of the domestic cat, which was nearly 100% (Section 3.3; $p < 0.05$).

No significant difference was detected in the plasma T_{max} or C_{max} of cheetahs and domestic cats ($p > 0.05$).

4.2.4. *Fraction absorbed*

Comparison between cheetahs ingesting a soy extract tablet and domestic cats administered with purified genistein and daidzein, of similar dose. The total fraction of ingested genistein dose detected in the plasma of cheetahs (33.8 and 33.6%) was significantly lower than the average fraction absorbed in domestic cats (53.9% $p < 0.05$). The total fraction of ingested daidzein dose appearing in cheetah plasma (9.13 and 10.5%) was also lower than that of domestic cats (29.2%, $p < 0.05$).

No significant difference was detectable between the fraction of genistein absorbed that appeared as a conjugate in cheetahs (32.1% or 32.3%) compared to domestic cats (58.4%). However, the fraction of absorbed daidzein that appeared as a conjugate in cheetahs was lower (8.28% or 9.53%) than domestic cats (30.5%; $p < 0.05$).

The fraction absorbed that appeared as unbound genistein and daidzein were both significantly higher in cheetahs (1.68% or 1.26% for genistein and 0.85% or 1.13% for daidzein), compared to domestic cats (0.36% for genistein and 0.25% for daidzein; $p < 0.05$; Section 3.3).

4.2.5. *Metabolite profile*

The presence or absence of detectable metabolite concentrations in the plasma of cheetahs and domestic cats following ingestion of soy extract tablets by both species is compared in Table 4.4.

	Cheetah	Domestic cat
Unbound genistein	+	+
Unbound daidzein	+	+
Genistein monosulphate	+	+
Daidzein monosulphate	+	+
Daidzein disulphate	+	+
Unbound equol	+	-
Equol monosulphate	-	+
Equol disulphate	-	-
O-DMA	+	-
Dihydrodaidzein	+	-

Table 4.4. Comparative metabolism of isoflavones in cheetahs and domestic cats, following ingestion of soy extract (both species). N = 2 cheetah and 6 domestic cats. Presence of detectable concentrations in the plasma is indicated by (+) whilst absence is indicated by (-)

4.2.6. Discussion

Although it must be noted that different dietary forms and background diets may have influenced the observed differences in isoflavone absorption, cheetahs appear to have a lower overall capacity to absorb genistein and daidzein as evidenced from plasma and faecal recovery. Aside from the previously mentioned dietary-related effects, this may related to their relatively short gastrointestinal tract compared to the domestic cat (Maskell and Johnson 1993; Sturgess *et al.* 2001; Cheetah Conservation Fund, pers. comm. 2006). Furthermore, cheetahs were deficient in their capacity to conjugate absorbed genistein and daidzein, in comparison to the domestic cat. This was

demonstrated in both the plasma and urinary conjugation profiles and is likely to explain the greater availability of unbound forms in the cheetah. Since the unbound forms have a higher affinity for the oestrogen receptors, these finding have critical importance for the potential influence of isoflavones in physiological functions of the cheetah. Although the reduced apparent absorption of isoflavones in cheetahs compared to cats may counteract this potential by reducing the total exposure of cheetahs to isoflavones, the relatively sparse sampling regime may have led to true absorption being underestimated. This is certainly supported in the case of daidzein in particular where a greater concentration of this isoflavone was recovered in the urine than detected in the plasma. Further investigation is therefore warranted with a larger sample size in order to elucidate the absorption and availability of isoflavones in the cheetah.

The capacity to produce equol from daidzein appears to be common to both cheetahs and domestic cats. Although further investigation is required in cheetahs consuming daidzein as part of their daily diet, this finding indicates similar microflora and/or enzymatic processes in the cheetah and domestic cat. However, cheetahs produced only equol in its unbound form, whilst domestic cats produced only equol monosulphate. This finding further suggests that the conjugation capacity of the cheetah may be reduced.

The detection of dihydrodaidzein and *O*-DMA in the plasma of cheetahs is in contrast to that of domestic cats consuming a comparable dose of isoflavones. A higher formation rate of sulphate conjugates in domestic cats may have prevented the further degradation of daidzein to forms such as *O*-DMA and dihydrodaidzein, which were consistently detected in the plasma of one cheetah but not that of domestic cats. However, domestic cats consuming daidzein as part of a commercially-prepared diet exhibited both these metabolites. Further investigation is necessary to determine if this disparity is a species- or dietary matrix-related effect.

Of interest is the observation that cheetahs consistently exhibited a second peak in the plasma concentration of detectable isoflavones. This may suggest that entero-hepatic recirculation of isoflavones is more prominent in cheetahs, compared to the cat. This is supported by the greater fraction of genistein recovered in the faeces of cheetahs, compared to domestic cats. However, it could also reflect alternative sites of absorption. Larger sample sizes and a more comprehensive sampling regime in the cheetah would be necessary to accurately compare these species.

Overall, faecal voiding of isoflavones appears to be greater in the cheetah, compared to the domestic cat. If this fraction was not the result of entero-hepatic recirculation, it is possible that hydrolysis and subsequent absorption of ingested isoflavones is inferior in the cheetah compared to the domestic cat. Indeed, this hypothesis is supported by findings of a reduced fractional recovery in the plasma of cheetahs compared to cats. This may occur as a result of differences in microflora, gut transit time, or enteric absorptive capacity between the species.

The meat-only diet of cheetahs may be responsible for some of the observed differences in metabolites and pharmacokinetics of isoflavones. The inclusion of carbohydrates in many commercially-prepared feline diets (Zoran 2002) such as those consumed by the domestic cats studied in Section 3 may have conferred domestic cats with a divergent gut microflora population and modulated fermentative capacity, compared to that induced by the meat-only diet of the captive cheetahs in this study. The greater production of hydrogen and short chain fatty acids following ingestion of carbohydrates may indicate enhanced fermentation processes (Uehara *et al.* 2001; De Boever *et al.* 2000; Rafii *et al.* 2003; Clavel *et al.* 2005; Decroos *et al.* 2005; Setchell and Cole 2006) and permit greater hydrolysis (and hence absorption) of ingested isoflavones by domestic cats. Investigation of the origin of the isoflavones detected in the faeces is necessary to determine the entero-hepatic recirculation and/or absorptive capacity in cheetahs.

4.3. Conclusions

- The cheetah is capable of absorbing, excreting and metabolising soy isoflavones. Equol production has been confirmed in the cheetah although further investigation is necessary to predict the proportion of the cheetah population capable of doing so.

- The cheetah may be reduced in its overall ability to absorb dietary genistein and daidzein, compared to domestic cats.

- The cheetah is capable of significant phase II metabolism of genistein and daidzein. However, the capacity of the cheetah to conjugate genistein and daidzein with sulphate moieties appears lower than that of the domestic cat. Although this may be a dose-related effect, the finding is important for captive cheetahs consuming isoflavones at the dose provided here.

- The availability of unbound genistein and daidzein is poor in the cheetah, compared to other non-felid species. However, compared to the domestic cat, the availability of these compounds is improved. This may confer greater opportunity for biological activity of these isoflavones in the cheetah than would be predicted in the domestic cat.

- Conjugation may be reduced in neonatal cheetahs. This indicates that feeding isoflavone-containing milk replacers to cheetah cubs may expose them to higher circulating concentrations of unbound isoflavones than would occur in adult cheetahs. This may have consequences for the potential for physiological perturbations of isoflavones in cheetah cubs. Further testing is warranted as other

causes for the apparently increased proportion of plasma unbound isoflavones include dose-response and chronic exposure.

- Investigation of isoflavone absorption, availability and metabolism in adult cheetahs consuming isoflavones as part of a part of a dietary matrix, and following chronic ingestion, is warranted.

CHAPTER FIVE:

Influence of Long-term Dietary Isoflavone Intake on Puberty Onset, Oestrous Cycle Characteristics, Reproductive Tract Histology and Sex Steroid Receptor Expression in Domestic Cats (*Felis catus*)

Results from Section 5.1 were presented at the Australian and New Zealand Nutrition Society Conference, December 2007. The abstract was published (included as Appendix 4) as: **Bell KM**, Ugarte CE, Tucker LA, Thomas DG. (2007). Genistein and daidzein do not affect puberty onset or oestrous cycle parameters in the domestic cat (*Felis catus*). *Asia Pacific Journal of Clinical Nutrition* 16(Suppl. 3): S72

Results from Section 5.2 were published as an abstract in the proceedings of the 16th International Congress of Animal Reproduction. The abstract is included as Appendix 5: **Bell K**, Ugarte CE, Tucker LA, Roe WD, Thomas DG. (2008). Assessment of reproductive histology and sex steroid receptor expression in the domestic cat (*Felis catus*) following chronic exposure to phytoestrogens. *Reproduction in Domestic Animals* 43(Suppl. 3). Pp 126.

5.0. Introduction

The captive cheetah population is not self-sustaining with only approximately 20% of captive animals being recorded as having bred successfully (Marker 2005). The hypothesis of Setchell *et al.* (1987ab) that dietary isoflavones may have been causative factors in the reproductive impairment of captive cheetahs consuming soy-containing diets remains unsubstantiated. However, to test this hypothesis an alternative animal model must be used due to the endangered status of the cheetah.

The findings of Chapter 2 suggest that isoflavone exposure of domestic cats and captive cheetahs may be considerable. Given that domestic cats and captive cheetahs consume dietary isoflavones in concentrations demonstrating physiological perturbations in other species (Chapter 2), and that a fraction of the ingested dose is absorbed (Chapter 3 and 4), it is feasible that these compounds exert biological activity in felids. However, mechanisms of isoflavone metabolism are efficient in cats (Chapter 3 and 4). Therefore, isoflavones are thought to have poor plasma availability compared to other mammalian species, which may alter the ability of isoflavones to influence reproductive parameters in cats.

This study aimed to determine the consequences of chronic dietary isoflavone ingestion on the onset of puberty, oestrous cycle characteristics, reproductive tract histology and sex steroid receptor expression in the domestic cat. To the author's knowledge, no previous study has been conducted to investigate these parameters in a felid species.

5.1. Experiment One: Effect of Genistein and Daidzein on Puberty Onset and Oestrous Cycle Parameters

5.1.1. Aim

This study had two objectives. Firstly, it aimed to assess whether the age and body weight at onset of regular oestrous cycling differed between cats chronically exposed to oral genistein and daidzein, and those without isoflavone exposure. Secondly, by monitoring oestrous cycle characteristics beyond the onset of regular cycling, it aimed to determine whether chronic exposure to oral genistein and daidzein modulated oestrous cycle length, regularity and/or vaginal epithelial cornification.

5.1.2. Materials and Methods

5.1.2.1. *Animals*

Fifteen female domestic cats were assigned to either the control group (n = 9) or treatment group (n = 8) at weaning (approx. 6 – 8 weeks). Two cats in the treatment group refused to eat the diet and were subsequently removed from the trial leaving n = 6. Siblings were split evenly (where possible) between the two groups. This trial was approved by the Massey University Animal Ethics Committee (2006).

Cats were bred and maintained at the Centre for Feline Nutrition (Massey University) for the duration of the trial. Cats were group-housed (maximum 9 per pen) in multi-level outdoor pens (approx. 5m x 2m x 3m), exposed to natural day/night cycles and provided

with daily exercise opportunities and environmental enrichment. Control and treatment groups were housed in adjacent pens. Each cat was weighed weekly and body weight recorded.

Kittens were reared by their mothers until 6 weeks of age, during which time they had access to the queen's diet (a commercial diet which met AAFCO standards for gestation and lactation). This diet was assayed to contain very low (16 μg total isoflavone/g DM) levels of isoflavone (see Section 2.1.3.1) and exposed the queens to a total isoflavone dose of approximately 0.56 mg – 0.84 mg/kg BW. Intake of the maternal diet by kittens prior to weaning was assumed to be minimal, but accurate assessment of the intake was not possible due to group housing with the queen. The duration of pre-weaning exposure was standardised across treatment and control groups. At 6 weeks of age, kittens were weaned from the queens and separated into treatment and control groups. Kittens were gradually adapted to the trial diets and the first day of sole consumption of the trial diet was recorded as the start of the trial for each individual cat.

5.1.2.2. *Dietary isoflavone administration*

The base diet for both control and treatment groups was a moist, feline diet, which met AAFCO requirements for growth in the domestic cat (see Table 3.1). This diet was assayed to contain no detectable isoflavones (methodology according to Section 2.1.2). The purified (99.9%) form of genistein and daidzein (LC Laboratories, MA, USA) was added to the base diet to provide a calculated dose of 300 μg total isoflavone/g dry matter (DM). This form of isoflavone administration has previously been determined to result in approximately 54% absorption into the plasma of ingested genistein dose and 29% absorption of daidzein (see Section 3.3.3.4). This dose was representative of high exposure through consumption of commercially prepared feline diets (see Section 2.1.3.1.). However, due to the small quantities of isoflavones to be added to large quantities of base diet it was necessary to use a vehicle carrier (freeze-dried base diet).

Batches of freeze-dried base diet (500 g) were prepared, containing 345 mg genistein powder and 345 mg daidzein powder. A 150 g aliquot of this mixture was added to 3 kg of base diet and mixed thoroughly in a food blender. The same concentration of vehicle material was added to the control diet, without the addition of isoflavone powders.

Cats were provided with food and water *ad libitum* during the trial, with food intake per group weighed on a daily basis. Monthly assessments were made of individual food intake by separation of each cat into individual metabolism cages for a 24 h period during which time food intake and urinary and faecal output were recorded.

Sub-samples of the two diets were assayed for isoflavone content at regular intervals throughout the trial according to methodology described in Section 2.1.2.

5.1.2.3. *Vaginal cytology*

A vaginal smear was taken from each cat three times per week, starting at three months of age. A moistened cotton swab was gently inserted into the vaginal vault of each cat, rotated and removed. The swab was then rolled across the surface of a microscope slide (Southern Veterinary Services, Palmerston North, NZ) and allowed to air dry. Each smear of exfoliated vaginal epithelial cells was fixed in ethanol and stained in sequential baths of eosin and polychrome (Gribbles Scientific Ltd., Palmerston North, NZ). After air-drying, smears were examined for the presence of parabasal, intermediate and nucleated or anucleated superficial cells under 40 x magnification (Olympus microscope, CX31 RTSF, Olympus Corp., Tokyo, Japan). A total of 100 cells were counted and the composition of this sample was used to determine the proportions of each cell type. The overall cell yield, amount of non-cellular debris and clumping of cells were recorded at 10 x magnification.

5.1.2.4. *Oestrous cycle parameters*

Smear scoring was based on the maturation index reported by Mills *et al.* (1979) to define the various stages of the oestrous cycle in the cat (see Figure 5.1). The other parameters of cell yield, clumping and debris were used to confirm maturation index scoring (Mowrer *et al.* 1975). Apparent inter-oestrus was defined from smears having > 5% parabasal cells and < 60% superficial cells (Figure 5.1.a). Apparent oestrus was defined from smears having < 5% parabasal cells and > 80% superficial cells (total nucleated and anucleated) (Figure 5.1.c.). Smears during this stage were also defined by the absence of non-cellular debris and high cell yield with multiple areas of cell clumping. Apparent anestrus periods were defined from persistence of consecutive smears with > 50% parabasal cells, for at least 20 days.

The age at which the first hormonally-induced cytological changes were detected was recorded as the onset of puberty for each cat. Since initial apparent oestrus periods were not consistently followed by regular cyclical changes in vaginal cytology, the first period of cornification that was repeated at least twice within a subsequent 20 day period, was also recorded and defined as the onset of regular cycling. Each apparent inter-oestrus period greater than 15 days in length, following the onset of regular cycling, was carefully examined for smears indicating either apparent pro-oestrus or metestrus, but without an intervening apparent oestrus period. In cases where such indicator smears were identified, it was assumed that the interval between sampling (1-2 days) may have resulted in the failure to detect oestrus. As such, the indicator smear was counted as an apparent oestrus event. The duration of each apparent oestrus period was taken as the day or consecutive days where oestrus was detected on vaginal smears. Apparent inter-oestrus period length was recorded as the period between the sampling day following the last smear where oestrus was apparent, until the sampling day immediately prior to the next apparent detection of oestrus.

Since pseudopregnancy is known to result in a delayed return to oestrus of between 20 – 40 days (Feldman and Nelson 1996), the occurrence of spontaneous ovulation was defined by apparent inter-oestrus periods of > 20 days in duration, during which no evidence of unsampled (missing) oestrus events were detected.

Cats were sampled up to a minimum age of 261 days and a maximum age of 502 days (depending upon date of birth and entry into the trial). Smears were obtained over a 421 day period, with the last smear obtained at a mean age of 435 days (± 15.97).

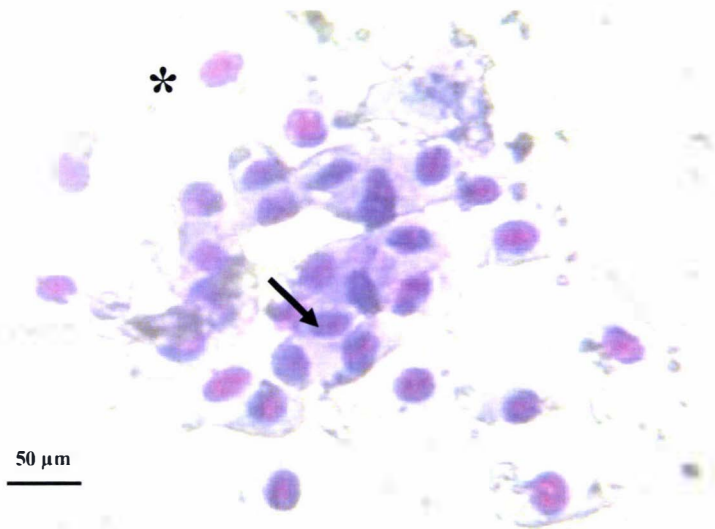


Figure 5.1a. Representative smear indicative of inter-oestrus. N.b. the predominance of parabasal cells (→), and relatively few intermediate cells (*). Eosin and polychrome stain.

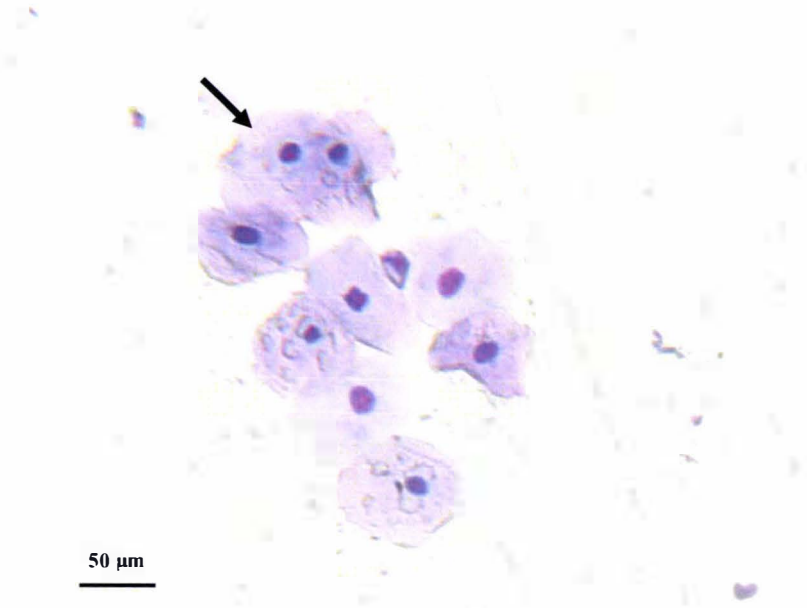


Figure 5.1b.
Representative smear indicative of pro-oestrus.
N.b. the predominance of nucleated superficial cells (→).
Eosin and polychrome stain.

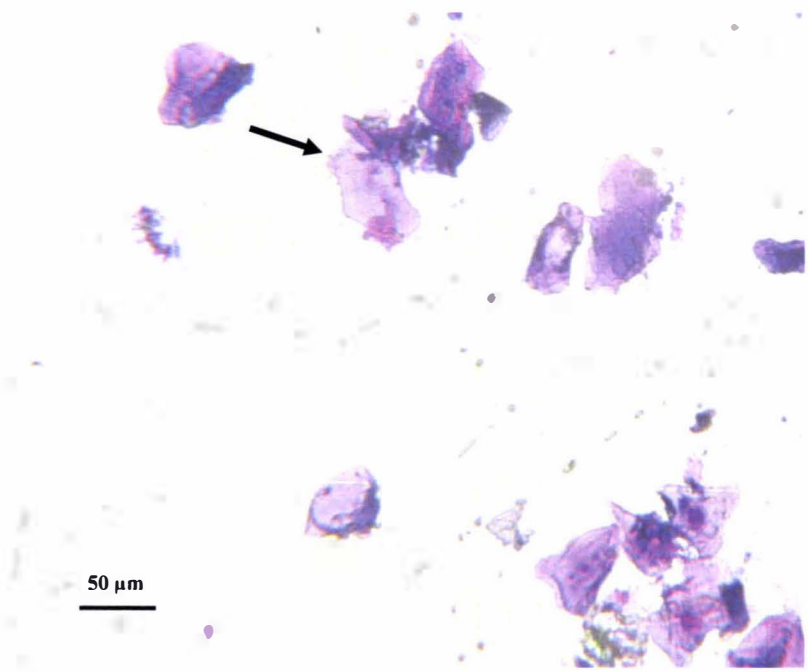


Figure 5.1c.
Representative smear indicative of oestrus.
N.b. the predominance of anucleated superficial cells (→).
Eosin and polychrome stain.

5.1.2.5. *Behavioural testing*

During the final four months of the trial, cats were opportunistically assessed for behavioural signs of oestrus at the time of vaginal smear sampling. Behavioural tests evaluated the presence or absence of the lordosis response (lifting of the hindquarters

when stroked along the spine in a dorso-caudal direction), lateral tail deviation when stimulated at the base of the tail and perineum region, treading by the hind legs and/or adoption of the mating posture when stimulated at the base of the tail and in the perineum region. Cats were observed prior to sampling for signs of rolling and rubbing with pen-mates. Husbandry staff were also requested to record the presence of rolling, vocalizing and excessive rubbing by each cat. Behaviours were scored as 1 if present or 0 if absent, and these were then summed to give a maximum score of 6 if all behaviours were detected.

5.1.2.6. *Statistical analyses*

Residual data were tested for normality with the Anderson-Darling test. Normally distributed data (i.e. puberty and the onset of regular cycling) were tested for differences between group using an ANOVA. Data that were not normally distributed (i.e. apparent oestrus and inter-oestrus length, time to attain regular cyclicity, nucleated and anucleated superficial cell counts) were examined with the Mann-Whitney test. Where data were not normally distributed, the median is reported instead of the mean (Glantz 2005). The incidence of spontaneous ovulation (defined in Section 5.1.2.4.) in the two groups was compared using a Fisher's exact test. Regression analysis was performed on the behaviour score *vs.* cytological detection of oestrus. The behaviour scores during both apparent oestrus and inter-oestrus periods were compared between groups using a Mann-Whitney test. All statistical procedures were carried out with Minitab software (version 15, Minitab Inc., PA, USA) with the level of significance set at $p < 0.05$. Data are presented as mean (± SEM), unless otherwise stated.

5.1.3. Results

5.1.3.1. *Isoflavone exposure*

5.1.3.1.a. *Feed intake and diet content*

Estimated daily feed intake of cats, calculated from the group total intake divided by the number of cats in the group, did not differ significantly from the daily intake of cats recorded when housed individually in metabolism cages overnight. Therefore, all subsequent analyses were performed on group-housed data, unless otherwise specified.

Average intake at specific ages could not be calculated due to the group housing of cats with large age differences. Mean daily feed intake was significantly lower in the treatment group (262.9 ± 22.2 g) compared to control cats (289.3 ± 23.5 g) ($p < 0.01$). When calculated on a metabolisable energy (ME) basis, treatment cats consumed an average of 60.9 kJ/d whilst control cats consumed 66.9 kJ/d. This difference was reversed when the final metabolic cage intake for each cat (prior to termination of the trial) was assessed. In this case control cats had a mean daily intake of 111.1 g (± 22.8) while treatment cats consumed an average of 157.4 g (± 43.9) ($p < 0.05$).

The mean total isoflavone content of dietary sub-samples taken throughout the trial was 270.6 (± 44.1) μg/g dry matter (DM) total isoflavones. The mean genistein content was 135.4 (± 22.5) μg/g DM and daidzein was 135.2 (± 29.7) μg/g DM. No isoflavones were detectable in any control diet sub-samples. The DM content of the diet was 21.9% and therefore the isoflavone dose, on an as-fed basis, was 59.2 μg total isoflavones/g, or 29.6 μg genistein/g and 29.6 μg daidzein/g.

5.1.3.1.b. *Body weight and ingested isoflavone dose*

The body weight (BW) of each cat was recorded on a weekly basis throughout the trial. Plotting the BW over time indicated that cats attained maximal body weight by approximately 300 days of age (evidenced by a plateau in the growth curve) (Figure 5.2.). The mean BW at 90 days, 180 days, 300 days and 400 days was then calculated for both groups (Table 1). Treatment cats were heavier than control cats at 300 days ($p < 0.05$) but this difference had reduced by 400 days ($p = 0.05$). No significant difference was detectable for BW between 300 days and 400 days, supporting the attainment of maximal body weight by 300 days. Although the control cats had an apparently slower rate of BW gain during the trial compared to the treatment cats, the rates of gain for all cats was within the normal range for the colony.

Age (d)	Control group mean, kg	Treatment group mean, kg
90	1.09 (± 0.06)	1.12 (± 0.05)
180	2.09 (± 0.05)	2.24 (± 0.08)
300	2.72 (± 0.08)**	3.00 (± 0.10)**
400	2.87 (± 0.06)*	3.19 (± 0.13)*

Table 5.1. Average body weight (± SEM) for cats at various ages. Significant inter-row differences are indicated by **p < 0.05 or *p = 0.05.

By the end of the trial treatment cats were consuming an average of 4.88 – 5.19 mg total isoflavones/kg BW/day, providing approximately equal doses of 2.44 – 2.56 mg/kg BW/day of genistein and daidzein.

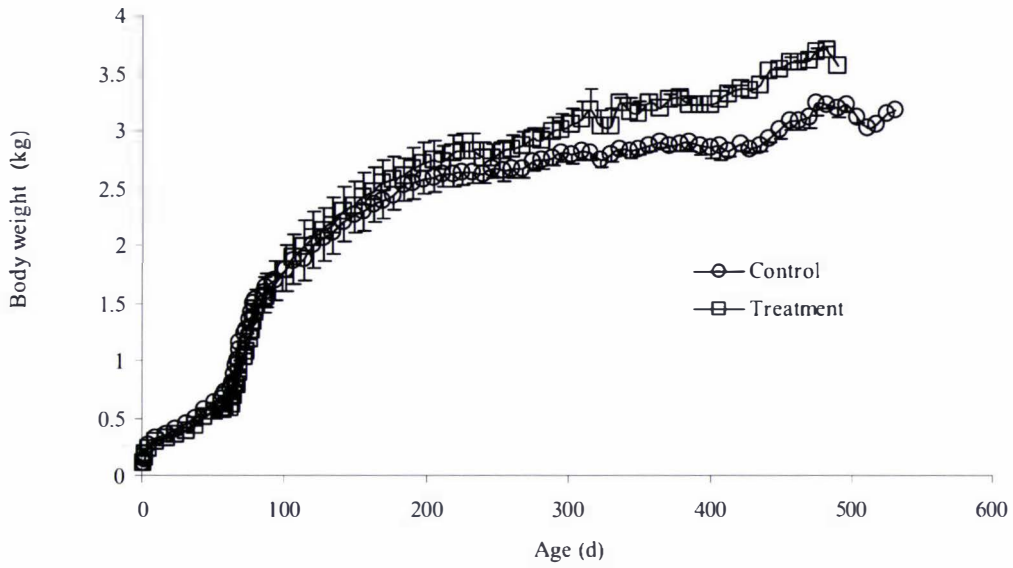


Figure 5.2. Mean weekly body weight (kg) of cats in the control (n = 9) and treatment (n = 6) groups, from birth until maturity. Error bars represent SEM.

5.1.3.2. *Age and BW at puberty onset*

The mean age at puberty was 195.0 (± 19.9) days in control cats, and 171.1 (± 26.6) days in treatment cats. No significant difference was detected between the two groups in this parameter. Body weight at the onset of puberty also did not differ significantly between groups (2.17 ± 0.19 kg in the control group and 1.85 ± 0.20 kg in the treatment group).

5.1.3.3. *Age and BW at onset of regular oestrous cycling*

Control cats had a mean age at onset of regular cycling of 241.9 (± 15.3) days, whilst the treatment group median was 206.0 (± 17.4) days. This difference was not statistically significant. Control cats weighed, on average 2.53 (± 0.14) kg at the onset of regular cycling, but this was not significantly different from treatment cats at the same stage (2.50 ± 0.22 kg). Although the period of time between puberty onset and the initiation of regular oestrous cycling was greater in treatment cats (56 days) compared to control cats

(14 days), the high degree of variation between cats meant this difference also did not achieve statistical significance. Figure 5.3 illustrates a representative cycle of parabasal cell appearance-disappearance over time in a control cat.

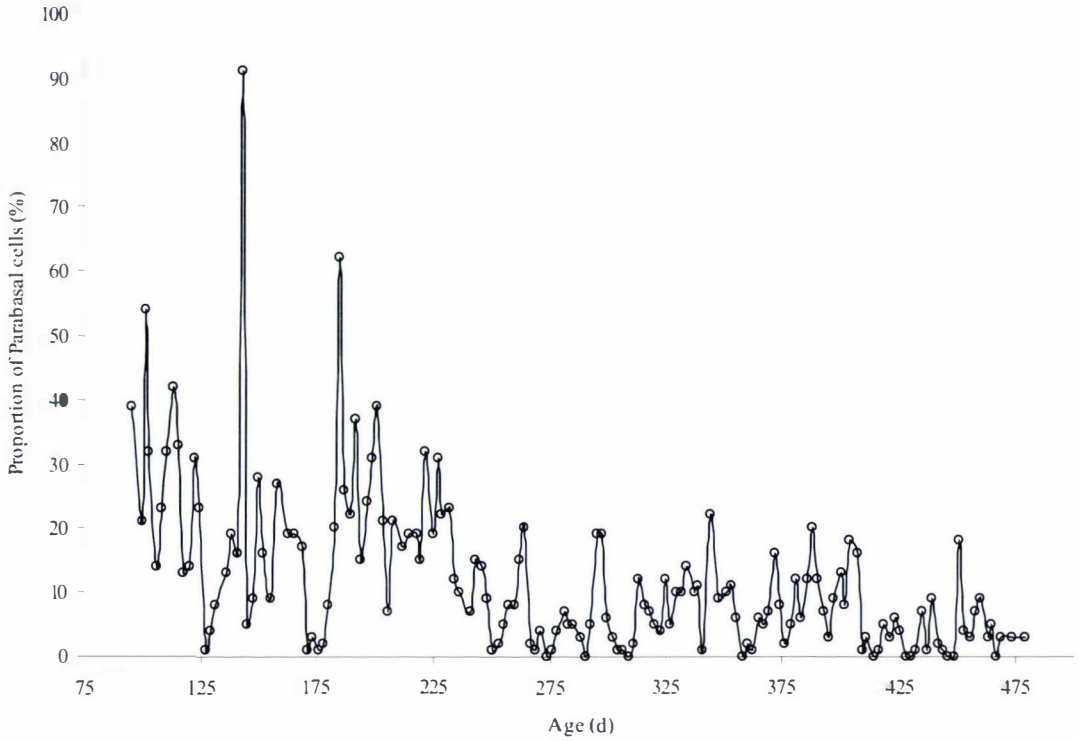


Figure 5.3. The proportion (%) of parabasal cells appearing in vaginal smears of a control cat over time.

5.1.3.4. *Length of apparent oestrus*

The average number of apparent oestrus events per animal did not differ between groups (17.4 ± 1.20 in the control group, compared to 17.7 ± 1.29 in the treatment group). No difference in apparent oestrus duration was observed between groups. A total of 157 oestrus-like events were recorded in the control group (median length of 1.0 day, min 1; max 23), and sixty nine oestrus-like events were recorded in the treatment group (median length of 1.0 day, min 1; max 14).

5.1.3.5. *Length of apparent inter-oestrus*

The median apparent inter-oestrus length (including all inter-oestrus periods) did not differ significantly between groups. The median apparent inter-oestrus length was then re-calculated for both groups following removal of periods indicative of spontaneous ovulation (inter-oestrus periods greater than 20 days), but still did not differ between groups. The apparent median inter-oestrus length was 7.0 days in both the control (min 1; max 18) and treatment (min 4; max 18) groups.

5.1.3.6. *Incidence of apparent spontaneous ovulation*

A total of 153 and 66 apparent inter-oestrus periods were recorded in the control group and treatment groups, respectively. The apparent inter-oestrus periods of the control group animals included 4.08% (6/147) extended inter-oestrus periods (of duration greater than 20 days). Treatment group inter-oestrus periods included significantly more ($p < 0.05$) extended inter-oestrus periods (13.85%, 9/65).

5.1.3.7. *Proportion of superficial cells*

In order to gauge vaginal cornification independently from the predicted stage of oestrus, the number of nucleated and anucleated cells in all smears were compared between groups, without cytological interpretation. The median number of nucleated superficial cells did not differ between groups and was 75 from both control and treatment cats (H = 0.01). The median anucleated superficial cells was 4 in both control and treatment cats (H = 1.42).

5.1.3.8. *Sexual behavioural demonstration*

Overall behaviour scores were significantly higher in the treatment group than control group (median 1.50 *vs.* 1.00, respectively, $p < 0.01$). Behaviour scores during apparent

oestrus periods appeared to be higher in the treatment group (median 2.00) compared to the control group (median 1.50; $p < 0.05$), as well as during apparent inter-oestrus periods (1.00 *vs.* 0.75). A trend was observed towards higher behaviour scores during apparent oestrus (as identified by vaginal cytology) than those observed during apparent inter-oestrus periods in the control group (1.50 compared to 0.75, respectively, $p < 0.10$). However, no significant difference could be detected between these periods in the treatment group ($p > 0.10$).

Behaviour score was not correlated with apparent stage of oestrous, as defined by vaginal cytology ($R^2 < 0.5$), in either group.

5.1.4. Discussion

The ability of genistein to influence feed intake has been demonstrated previously (Cave *et al.* 2007bc), and the finding of a reduced overall daily feed intake by treatment cats, compared to control cats in this study is in agreement with previous findings in overweight domestic cats (Cave *et al.* 2007b). However, the previous studies included a dose of 100 mg/kg BW, which is much higher than the dose used in this study (< 3 mg/kg BW). Since both studies utilised a purified form of genistein, the lower effective dose in this study may be due to the extended duration of exposure of cats in this study, compared to the 5-day exposure in the earlier study (Cave *et al.* 2007b). The bioavailability of genistein appears to have been lower in the overweight adult cats used by Cave *et al.* (2007b) compared to the plasma fraction of cats exposed to doses closely resembling those used here (Section 3.3) and may therefore explain the apparently lower effective dose observed in the present study. Differences may also be explained by temporal and/or age factors, although the use of gonadectomised cats by Cave *et al.* (2007b) may also have modulated isoflavone activity. The modulating effects of endogenous oestrogens have been reported for other isoflavone-related physiological endpoints (Section 1.4.4.3) and may explain the differences observed in the entire

females used in this study. Further, the inclusion of daidzein in the dietary exposure regime of this study is likely to have contributed to the observed effect.

However, dietary isoflavones appear to have exerted only a transient effect on BW in this study. Isoflavone-exposed cats were heavier than control cats at 300 days of age, despite a lower overall feed intake. However, the difference in BW was only marginally detectable beyond this age ($p = 0.05$). This indicates that the difference observed in group feed intake was either insufficient to exert consistent BW changes, or that body composition was modified without concurrently altering total BW. Cave *et al.* (2007c) demonstrated an increase in lean body mass and reduced fat accumulation in domestic cats exposed to oral genistein following gonadectomy, without accompanying changes in total BW. The occurrence of similar changes in body composition in cats consuming isoflavones may also have occurred in the current study, although this was not measured. Further testing with increased sample sizes would be required to elucidate this potential effect. It must also be noted that feed intake was only lower in treatment cats when group-housed data was compared over all ages. When the individual animal intakes were compared using metabolism-cage derived values prior to termination of the trial (i.e. at the oldest age point for all cats) the pattern was reversed so that treatment cats were found to consume greater amounts than control cats. It is therefore possible that an alternative explanation exists such that isoflavone effects on feed intake are age-dependent. Full investigation of the effect of isoflavones on cat feed intake was not an objective of this study and as such its design did not permit further exploration of this hypothesis.

Odum *et al.* (2004) demonstrated that rats fed diets of varying energy content exhibited uterotrophic changes and altered puberty onset, independent of isoflavone and exogenous oestrogenic compound administration. However, neither accelerated puberty onset, nor uterotrophic effects (see following Section 5.2.3.1) were observed in cats during this study. As such the difference in energy intake between groups in this study is unlikely to have been a confounding variable. Body weight is also known to be an important

predictor of puberty onset in domestic cats (Arthur *et al* 1989; Feldman and Nelson 1996) but body weight was unaffected by isoflavone exposure at the time of puberty onset in the study cats.

The low sample size and the lack of external validation of the vaginal cytological indicators used to ascertain oestrus (i.e. through concurrent serum hormone analysis) are potential problems in this study. It is possible that apparent oestrus events in treatment cats were not true oestrus events, but instead reflective of increased cornification of vaginal epithelia in response to isoflavone treatment. Therefore oestrous cycling may actually have been suppressed following isoflavone exposure, but would have gone undetected in this study due to the potentially erroneous measurement of oestrous cycling. However, evaluation of the smears without interpretation of the apparent stage of oestrous demonstrated that the proportion of superficial cells in smears was not significantly different between groups. This indicated that isoflavones did not cause a detectable increase in the cornification of vaginal epithelium of treatment cats, under these study conditions. The finding that apparent oestrus cycle length, and the average number of apparent oestrus events per animal were not significantly different between groups provides further evidence to support the interpretation of vaginal cytology data. Although no significant correlation could be detected between apparent stage of oestrous and behaviour score, this is more likely to be a consequence of the opportunistic collection of behaviour data, and small sample sizes, rather than reason to dispute the reliability of vaginal cytology.

Chronic exposure to genistein and daidzein, at doses achievable from feline diets available in New Zealand (approximately 5 mg/kg BW) (Bell *et al.* 2006) did not alter puberty onset, the age of first regular cycling, or the apparent oestrus or inter-oestrus length in domestic cats. It is likely that the lack of observed effects is a consequence of the poor oral bioavailability of these compounds in cats (see Section 3.3.3; Cave *et al.* 2007a). These findings are consistent with that of Cave *et al.* (2007c) in which a dose of

100 mg genistein/kg BW was required before changes in vaginal cytology could be detected in ovariectomised cats.

However, other physiological parameters were observed to be modulated by isoflavone exposure. It is possible that isoflavones caused a delay in the onset of regular cycling following puberty. Isoflavone-treated cats typically took longer to begin regular cycling, once puberty was confirmed cytologically than control cats. Although the large difference was not statistically significant, this may primarily be a consequence of the small sample size and inter-individual variability. Further testing with larger samples sizes is warranted.

The increase in incidence of apparent spontaneous ovulation observed in this study is a previously unreported potential consequence of isoflavone exposure. However, previous studies reporting modulation of the reproductive system by dietary isoflavones typically include only rhythmic or cyclic ovulatory species. This is the first long-term study of isoflavone impacts in a felid species, in which ovulation is generally induced by vaginal stimulation. Previous investigations of isoflavone activity in felids have used shorter exposure regimes and/or did not evaluate characteristics of the oestrous cycle (Setchell *et al.* 1987ab; White *et al.* 2004; Cave *et al.* 2007b,c,d).

Both domestic cats and cheetahs are known to undergo periods of pseudo-pregnancy as a result of non-fertile mating and/or spontaneous ovulation (Asa *et al.* 1992; Gudermuth *et al.* 1997). Non-mating stimuli (e.g. female-female mounting and/or non-tactile presence of a male) are generally thought to induce these ovulation events (Brown *et al.* 1996a). However, treatment and control cats were housed under identical conditions and therefore had equal exposure to these factors. Therefore, the increased incidence of apparent spontaneous ovulation in treatment cats is not thought to be due to non-mating stimuli. Likewise, vaginal sampling of both treatment and control group animals was identical and does not provide an explanation for the between-group differences. However, it is

possible that isoflavone exposure caused a heightened sensitivity to vaginal stimulation, and indirectly resulted in the greater number of spontaneous ovulation events observed.

Induced ovulation in the domestic cat and cheetah is thought to increase the opportunity for conception during the receptive stage of the female reproductive cycle, since a female will continue to cycle repeatedly until ovulation takes place. Oocytes are, therefore, conserved for periods when sperm is likely to be present in the reproductive tract. Ovulation in the cat triggers an endocrine environment which serves to prepare the uterus for implantation. If ovulation does not coincide with conception, a period of pseudo-pregnancy ensues. This delays the cat's return to oestrus, thereby reducing her chance of conceiving within a given breeding season. Moreover, since a finite number of oocytes are produced in the female ovary, frequent ovulation of oocytes by unmated females may substantially reduce the lifetime capacity for fertile ovulations. The ability of dietary isoflavones to initiate ovulation in these induced ovulators may effectively reduce the fertility of cats. A similar situation has been proposed for isoflavone-exposed rodents exhibiting multi-oocyte follicles, such that ovulation of follicles containing more than one oocyte may lead to the premature depletion of oocytes in later life (Jefferson *et al.* 2002a). A further potential consequence of an increased incidence of spontaneous ovulation is the greater risk of the onset of pyometra (Agudelo 2005), which also has important long term health and fertility implications.

The hypothalamic region of the brain is central to sexual behaviour expression in females. Oestrogen receptor-α (ERα) is critical in mediating this behaviour and oestrogenic compounds are known to enhance sexual behaviour (e.g. the lordosis response), in treated females (Patisaul *et al.* 2001; Kouki *et al.* 2003). Lordosis has been shown to be reduced in rodents following isoflavone exposure, and this is thought to be due to an anti-oestrogenic effect of isoflavones on ERα- and ERβ-dependent gene expression in the hypothalamus (Patisaul *et al.* 2001; Kouki *et al.* 2003; Kudwa *et al.* 2007). An increase in sexual behaviour was observed for isoflavone-exposed cats in this study, independent of the apparent stage of oestrus. This finding, although requiring

validation, may indicate isoflavone interference at the level of the hypothalamus. It is possible that isoflavones caused hyper-expression of sexual behaviours, without concurrent cytological detection of oestrus, so that the stage of oestrous was less easily defined by changes in behavioural scores in treatment cats, compared to control animals. However, behavioural data was collected opportunistically at the time of vaginal smear sampling, and was not a primary aim of this study. More extensive studies, including larger sample sizes, more strictly controlled behavioural testing, and determination of ERα expression in the hypothalamus, would be necessary to evaluate this hypothesis.

5.2. Experiment Two: Effect of Genistein and Daidzein on Reproductive Tract Histology and Sex Steroid Receptor Expression

5.2.1. Aim

This study aimed to determine the influence of chronic oral exposure to genistein and daidzein on morphological and histological parameters and sex steroid receptor expression in the reproductive tract tissue of adult female domestic cats.

5.2.2. Materials and Methods

5.2.2.1. *Animals*

Twelve (8 control and 4 treatment group) domestic short-haired female cats were available for use in this study. The same animals included in Section 5.1.2.1 were included with the exception of 3 animals. One control cat was lost to Feline Infectious Peritonitis. Two cats were randomly eliminated from the treatment group due to unplanned pregnancies following the accidental release of an entire male cat into their enclosure during routine cleaning procedures by colony staff. Feed intake and isoflavone exposure are described in Section 5.1.2.1. and 5.1.2.2.

5.2.2.2. *Reproductive tract collection*

All females were defined to be in the inter-oestrus phase of their oestrous cycle at the time of reproductive tract collection. This was confirmed using cytological assessment

prior to surgery by vaginal smears obtained for two days immediately prior to surgery. This trial was approved by Massey University Animal Ethics Committee (2006).

Average age at tissue collection was 428 (± 22.05) days. The presence of vaginal smears that were indicative of inter-oestrus was used as the main criteria to determine tissue collection, rather than any standardisation by age. One female in the treatment group developed a suppurative endometritis (pyometra), requiring early surgical removal of the reproductive tract at 267 days of age. Cats had consumed the trial diets for an average of 394 days (± 22.04) at the time of tissue collection.

Females were fasted for 10 h prior to surgery and ovario-hysterectomy, performed by a qualified veterinary surgeon. Anaesthesia was induced by sub-cutaneous injection of Zoletil 100 (500 mg/ml each of tiletamine and zolazepam, 12 mg/kg BW) (Virbac, Auckland, NZ) prior to the animal being intubated and maintained on halothane/oxygen for the duration of the procedure. The animal was positioned in dorso-ventral recumbancy and an incision made in the midline to expose the reproductive tract. The left and right side of the reproductive tract were identified and tagged prior to removal from the abdominal cavity. The uterus was ligated and excised immediately anterior to the cervix and the entire tract then trimmed of fat and wet weight recorded. The incision was sutured closed and the cat allowed to recover in a metabolism cage for 14 days. Analgesia was provided via temgesic (324 μg/ml buprenorphine hydrochloride, 0.03 mg/kg BW) (Reckitt Benckiser, Auckland, NZ) administered by sub-cutaneous injection, post-operatively and ketofen (ketobrofen,1 mg/kg BW) administered *per os* for 2 days following surgery. The suture line was examined daily and sutures were removed at 14 days post-operation. The cats were then returned to normal housing at the colony.

5.2.2.3. *Reproductive tract processing*

After weighing, the reproductive tract was dissected into 3 sections (ovaries, uterine horns and uterine body). The ovarian surfaces were examined for the presence of visible follicles, corpora lutea, and corpora haemorrhagica, and the number of each was recorded. Each section of reproductive tract was then measured accurately with Vernier callipers and weighed. The ovaries were dissected in the sagittal plane, along the midline and half of each organ transferred to 10% neutral-buffered formalin (NBF), while the other half was snap-frozen in liquid nitrogen. The uterine horns were dissected transversely at the mid-point, and the anterior section fixed in NBF whilst the posterior section was snap-frozen in liquid nitrogen. The uterine body was dissected transversely, and half was fixed in neutral-buffered formalin and the other half snap-frozen in liquid nitrogen. The snap-frozen samples were stored as back-up samples in a -80°C freezer.

Neutral-buffered formalin fixed samples were processed on a Leica® TP1050 Tissue Processor and dehydrated through graded ethanols (70%, 95% and absolute ethanol) (Becton Dickinson, Poole, UK) at an ambient temperature. Samples were then cleared in xylene and impregnated with Paraplast® Tissue embedding Medium (Global Science and Technology, Auckland, NZ), under pressure at 60°C. Samples were then embedded using a Leica Histo Embedder (Global Science and Technology, Auckland, NZ) and cut using MicroTec® Rotary Microtome (Global Science and Technology, Auckland, NZ), and Feather S35 disposable blades (Global Science and Technology, Auckland, NZ), at 3 μm (for haematoxylin and eosin (H&E) staining), or 5 μm (for immunohistochemistry (IHC) staining,). The sections were then floated onto a Thermo® Tissue Bath (Medica Pacifica, Auckland, NZ) at 43°C.

Sections for histology were mounted onto Superfrost, precleaned slides and sections for IHC were mounted onto Superfrost PLUS, precleaned slides (Menzel-Glazer, Braunschweig, Germany). The slides for general histology were placed in a 60°C oven for 20 min, then stained with H&E using a Leica® Autostainer XL (Global Science and Technology, Auckland, NZ). Following staining and dehydration, the slides were

coverslipped on a Leica® CV5030 cover slipper (Global Science and Technology, Auckland, NZ) using Entellan® rapid mounting medium (Merck, Palmerston North, NZ). The IHC slides were placed in the 60°C oven for 1 h then removed and cooled.

5.2.2.4. *Histological assessment*

The uterine luminal epithelial cell height (LEH) was measured in 20 separate cells from each cat. Computer software (ImageJ, version 1.38. Rasband 2007; Research Services Branch, National Institute of Mental Health, MD, USA) was used to measure cell height. The numbers of follicles, classified as primordial, secondary, tertiary mature or atretic (Figure 5.4), were recorded for each ovarian section.

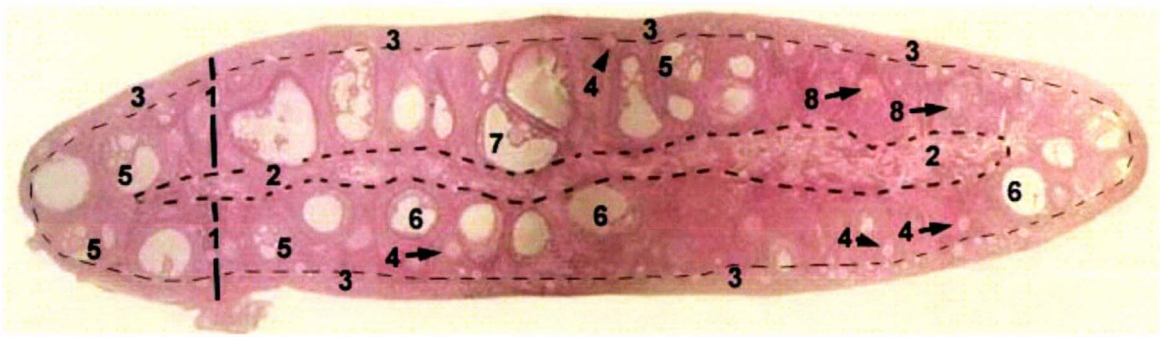


Figure 5.4. The ovarian structure classification. 1. Medulla. 2. Cortex. 3. Area of primordial follicles. 4. Primordial follicle. 5. Secondary follicle. 6. Tertiary follicle. 7. Mature follicle. 8. Atretic follicle. Stained with haematoxylin and eosin (Gunin 2007).

Haematoxylin and eosin stained slides were examined by a veterinary pathologist, who was blinded to the treatment groups. Prepared slides were examined for general morphology, broad classification of epithelial cells (glandular and luminal epithelium) and presence or absence of inflammatory cells. Ovaries were screened for abnormalities. Epithelial cells were classified as follows: elongated cuboidal cells that were broader than they were high were termed "attenuated cuboidal", slightly elongated cells with round nuclei were termed "low cuboidal" and generally square cells with round nuclei were termed "cuboidal". Rectangular cells with oval nuclei, where the height of the cell

was less than twice the height of the nucleus were termed "low columnar", whilst rectangular cells with oval nuclei, where the height of the cell was approximately twice that of the nucleus were termed "medium columnar". Rectangular cells with oval nuclei, where the cell height was greater than twice that of the nucleus were termed "columnar".

5.2.2.5. *Immunohistochemical (IHC) assessment*

Immunohistochemistry staining methods were used to assess oestrogen receptors (ERα and ERβ) and progesterone receptor (PR) levels in feline reproductive tissue (adapted from Martin de las Mulas *et al.* 2000). The ERα, ERβ and PR were identified using monoclonal mouse antibodies, IgG1 (NCL-ER-6F11 and NCL-ER-beta, Vision Biosystems, Victoria, Australia) and PR4-12 (Merck, Palmerston North, NZ), respectively, which were validated for use in feline tissue (manufacturer's statement).

Tissue sections, mounted on slides coated with *poly*-L-lysine (Sigma Chemical Co., Auckland, New Zealand) were deparaffinised by immersion in two xylene baths for 5 min each. Sections were then rehydrated in graded ethanol washes (absolute ethanol, 90%, 70% and 40% ethanol and then distilled water for 2 min each). Sections were transferred to pre-heated (boiling) citrate buffer (10 mM citric acid, pH 6.0) and microwave-heated for 5 min (HIGH power, 750 W microwave oven). The buffer volume was then adjusted to account for evaporation, before further heating at the same setting for 2 min. The buffer solution was then allowed to cool to approximately 45°C before slides were transferred to phosphate buffer solution (PBS) (pH 7.2) at room temperature. Sections were washed for 1 min in 3 separate PBS baths.

Slides with mounted sections were then removed from the PBS bath and tapped dry, and any excess moisture in the immediate area surrounding each section was removed with tissue paper. A liquid-proof ring (PAP pen, Invitrogen Life Technologies, Auckland, New Zealand) was then drawn around each section. Non-specific binding sites in the

tissue were blocked by adding 100 µl of 10% bovine serum albumin (BSA) (Roche Diagnostics, Mannheim, Germany) in 10% ovine serum and PBS to each section. Sections were incubated at room temperature in a humidity chamber for 45 min, before the blocking solution was removed and the primary antibody applied (ERα, ERβ or PR) at optimal dilutions, confirmed from preliminary studies (1: 50 for ERα and ERβ, 1: 30 for PR). Primary antibody was not applied to negative control tissue, which received 10% BSA solution instead. Sections were incubated overnight at room temperature.

Visualisation of antibody binding was achieved through the incubation of sections with a secondary antibody and a fluorescent marker. Following primary antibody incubation, sections were washed three times (1 min each time) in blocking solution. A 100 µl aliquot of the secondary antibody (biotinylated goat, anti-mouse IgG (Invitrogen Life Technologies, Auckland, NZ), reconstituted at 2 mg/ml, was then applied to each section, and incubated for 1 h in a humidity chamber at room temperature. Sections were then washed three times in blocking solution, before a streptavidin, fluorescent marker (diluted 1:20) was added (Alexa Fluro 546 conjugate; Invitrogen, Auckland, NZ). The slides were incubated for a further 1h at room temperature, in a humidity chamber. Cold tap water was run over the slides to wash off the streptavidin before slides were removed from the water and a drop of haematoxylin added to counterstain each section for 20 sec. Excess haematoxylin was then washed off using cold tap water.

Sections were dehydrated in a series of 70%, 90%, and 100% ethanol washes of 1 min each and after tapping dry, slides were washed in two separate xylene baths, for 1 min and 4 min, respectively. The area around each section was quickly dried, and a drop of Entellan mounting fixative (Merck, Palmerston North, New Zealand) applied over each sections. Coverslips were then applied and slides were left to air dry.

Positive control tissues (cat uterine tissue from control animals in the study, and human breast cancer tissue) were incubated with each of the 3 primary antibodies, and processed

according to the methodology described above within each experiment. In addition, 2 sets of negative control tissues were included in each experiment, to assess non-specific staining. The first negative control was a section from each cat which had the primary antibody substituted for blocking solution, and the second was 3 non-reproductive tissues (feline spleen, lung and heart tissue) known to be devoid of expression of the primary receptors under investigation, were processed with primary antibody, in each experiment.

Sections were examined under 40 x magnification on a Nikon Eclipse E600 microscope with epi-fluorescence illumination at 488 nm. Images were captured on a Nikon Digital Sight camera with NIS-Element software (Coherent Scientific, Hilton, Australia) and analysed for fluorescence-staining intensity and extent using Java-based image processing software (ImageJ, version 1.38. Rasband 2007; Research Services Branch, National Institute of Mental Health, MD, USA). A total of 100 individual cells were scanned within each tissue type in each section. Identical light intensities and capture settings were used for all sections. The level of light staining intensity detected in the negative control feline uterine tissue was set as the threshold of background luminescence in order to define positive staining in all test sections. The intensity of staining (colour-bits) was measured in positively-stained cells and then compared between cats as an estimate of receptor content within each cell. The proportion of cells staining positive *versus* those staining negative (below the negative control threshold) was assessed between animals as an estimate of receptor expression within the tissue type.

5.2.2.6. *Statistical analyses*

Residual data was tested for normality using the Anderson-Darling test. Data that was not normally distributed and did not have equal variance (morphometric data except body weight, luminal epithelial cell height, ERα staining intensity and distribution in the ovarian cortex) was tested for differences between groups using the Mann-Whitney test. Where data was not normally distributed, the median is reported instead of the mean (Glantz 2005). All other parameters were normally distributed and tested for between-

group differences with an ANOVA. Regression analysis was performed on potential confounding variables (age, BW, number of cycles prior to tissue collection and age of regular cycling onset) to determine their influence. To determine receptor distribution, the proportion of each receptor located in the various regions of each tissue was calculated by dividing the regional count by the whole-tissue count of the receptor-positive cells for each cat. This data was then tested for difference using the Mann-Whitney test. All statistical tests were performed with Minitab (version 15, Minitab Inc., PA, USA), with the level of significance set at $p < 0.05$. Data are presented as mean (± SEM), unless otherwise stated.

5.2.3. Results

5.2.3.1. *Morphological parameters*

No significant differences were detected in morphometrics (body length, limb length, thoracic circumference). Likewise, no significant differences were observed between groups in the wet weight of the reproductive tract (as a whole organ or dissected into separate components), or in the length or diameter of the tract components (Table 5.2). The cat with a diagnosed pyometra was excluded from this analysis.

	Control group	Treatment group
Entire tract weight (g)	2.13 (1.30 – 2.50)	1.90 (1.89 – 2.67)
Left uterine horn weight (g)	0.61 (0.38 – 0.84)	0.58 (0.46 – 0.81)
Left uterine horn length (cm)	3.48 (3.05 – 4.32)	2.96 (2.85 – 5.42)
Left uterine horn width (cm)	0.55 (0.05 – 0.68)	0.34 (0.05 – 0.60)
Right uterine horn weight (g)	0.58 (0.30 – 0.69)	0.61 (0.50 – 0.69)
Right uterine horn length (cm)	3.73 (3.16 – 4.52)	3.29 (3.15 – 4.55)
Right uterine horn width (cm)	0.53 (0.04 – 0.60)	0.42 (0.04 – 0.55)
Uterine body weight (g)	0.10 (0.02 – 0.16)	0.16 (0.13 – 0.17)
Left ovary weight (g)	0.12 (0.10 – 0.55)	0.14 (0.12 – 0.14)
Left ovary length (cm)	0.97 (0.84 – 1.20)	0.94 (0.90 – 1.00)
Left ovary width (cm)	0.54 (0.05 – 1.05)	0.51 (0.04 – 0.52)
Right ovary weight (g)	0.12 (0.08 – 0.15)	0.10 (0.09 – 0.15)
Right ovary length (cm)	0.92 (0.10 – 1.11)	0.85 (0.85 – 0.94)
Right ovary width (cm)	0.47 (0.05 – 0.61)	0.50 (0.05 – 0.55)
Body length (cm)	49.0 (47.0 – 55.0)	51.5 (48.5 – 54.5)
Right hind limb length (cm)	21.3 (20.5 – 23.5)	21.5 (20.5 – 23.1)
Right fore limb length (cm)	19.0 (13.0 – 21.0)	19.0 (13.5 – 19.5)
Cranial thoracic circumference (cm)	30.5 (29.5 – 38.0)	31.0 (28.5 – 33.0)

Table 5.2. Morphometric parameters expressed as median (minimum – maximum). All data from the control group cats (n = 8) is compared to healthy treatment group cats (n = 3). One treatment group cat was excluded due to diagnosis of a pyometra and early collection of data. No significant differences were detected between groups.

5.2.3.2. *Histological parameters*

No potentially confounding variables tested (age, BW, number of cycles prior to tissue collection and age of regular cycling onset) were found to be significant ($R^2 > 0.5$).

5.2.3.2.a. *Histopathology*

All reproductive tracts from the healthy cats were considered to be within the expected ranges of inflammatory cell infiltration. In general, few or moderate numbers of lymphocytes were present in the submucosal stroma, with most of these cells being found immediately beneath the luminal epithelium. In many cases there were low numbers of lymphocytes within luminal epithelial cells. The majority of tracts also had low numbers of neutrophils present within the submucosal stroma. The treatment cat diagnosed with suppurative endometritis (pyometra) had greater inflammatory cell infiltration than all other cats.

Most tracts had focal areas of acute haemorrhage in the supporting ligaments (mesometrium), which were considered to be a direct consequence of ovario-hysterectomy. Submucosal gland contents (eosinophilic secretions) were noted when present. No obvious pathology was detected in the ovarian tissue from any cat.

Each cell shape was assigned a numerical score and the overall pathologist's assessments were used to compare the predominant cell shapes of the uterine epithelium (luminal and glandular). The same process was undertaken to compare the presence of inflammatory cells and glandular secretion. No difference was detected in cell shape between groups for uterine tissue sections. Uterine luminal epithelial cells were predominantly low cuboidal, while glandular epithelial cells were low-medium columnar in shape. No difference was detectable between groups in the luminal epithelium shape of uterine horns (predominantly low cuboidal). However, treatment cat uterine horn glandular epithelium was more columnar (predominantly medium-columnar) in shape, compared to control cats (predominantly low columnar). This was significant ($p < 0.05$) in the right uterine horn, but not in the left uterine horn. However, combining epithelial scores for all uterine tissue within groups, resulted in no detectable between-group differences in overall uterine epithelial cell type score. No differences were detectable between groups in the presence of inflammatory cells or glandular secretion.

One cat in the treatment group was diagnosed with pyometra at 267 days of age. Premature removal of the reproductive tract of this cat was necessary due to the inflammatory response observed on vaginal smears. The cat exhibited a persistent mucoid vaginal discharge for 10 days prior to gonadectomy, and the presence of increasing concentrations of neutrophils and macrophages in her vaginal smear necessitated gonadectomy. Mucoid discharge began 7 days following her last apparent oestrus period. Prior to this, the cat had cat demonstrated an apparent inter-oestrus period of excessive duration (33 days), which was indicative of spontaneous ovulation (see Section 5.1.2.4). The cat showed no other signs of ill-health but was administered a precautionary 5 day course of post-operative antibiotics (Clavulox; clavulanic acid and amoxicillin, 12.5 mg/kg BW twice daily). Histological staining of sections from this cat's uterus demonstrated extensive glandular activity, typical of the luteal phase reported in healthy cats (Figure 5.5; Chatdarong *et al.* 2005). Cytological determination of oestrous stage was prohibited by the excessive presence of mucoid discharge and inflammatory cells in vaginal smears.

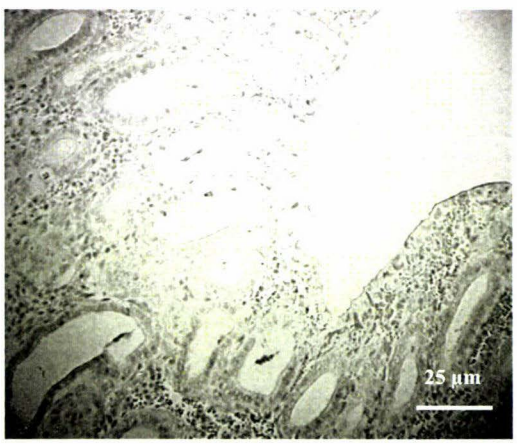


Figure 5.5a. The uterine lumen of the treatment cat exhibiting suppurative endometritis. N.b. the extensive glandular activity. Stained with haematoxylin and eosin.

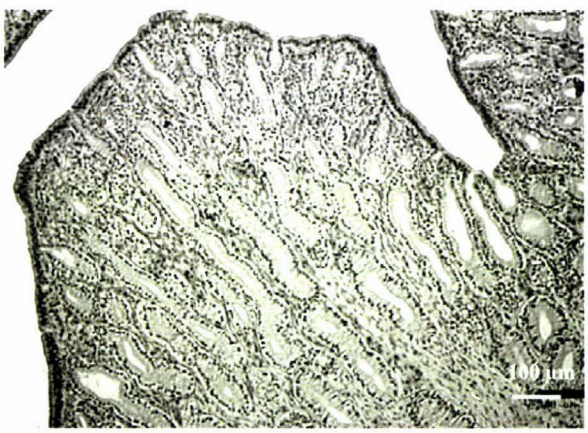


Figure 5.5b. Uterine lumen of a cat during the luteal phase. Stained with haematoxylin and eosin (from Chatdarong *et al.* 2005).

5.2.3.2.b. *Luminal epithelial cell height*

Uterine luminal epithelial cell height (Figure 5.6) was significantly greater in treatment cat sections (median 7.62 μm) compared to control cat sections (median 6.50 μm) ($p < 0.01$).

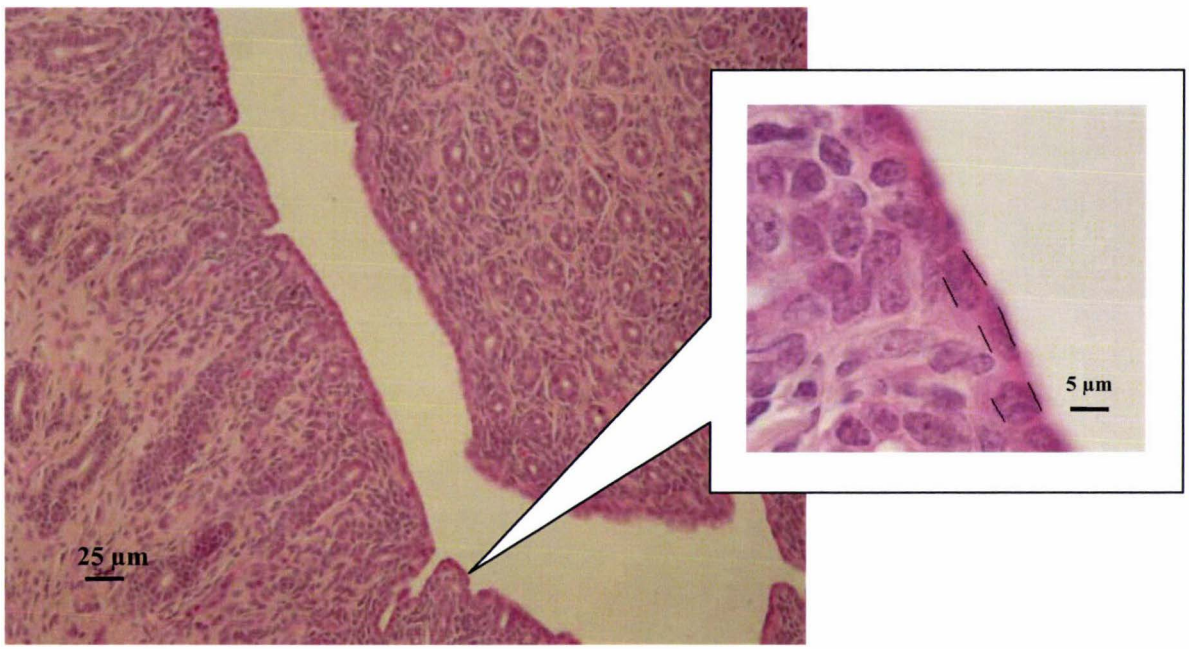


Figure 5.6. Section of the uterine lumen, stained with haematoxylin and eosin. Inset box shows magnification of the luminal epithelium with lines demarcating apical and basolateral surfaces for luminal epithelial cell height measurements.

5.2.3.2.c. *Ovarian follicular distribution and classification*

No significant difference was detected between groups in the number of primordial, secondary, tertiary, mature or atretic follicles (Figure 5.7). The number of corpora lutea also did not vary significantly between groups. This data is provided in Table 5.3.

	Control Group	Treatment Group
Primordial follicles	295.5	198.0
Secondary follicles	10.0	8.00
Tertiary follicles	7.00	4.00
Mature follicles	1.50	2.00
Atretic follicles	1.00	0.00
Corpora lutea	1.50	3.50

Table 5.3. Comparison of the mean number of ovarian follicles observed in ovaries from control (n = 8) and treatment cats (N = 4) at the time of ovario-hysterectomy. No significant differences were detected between groups.

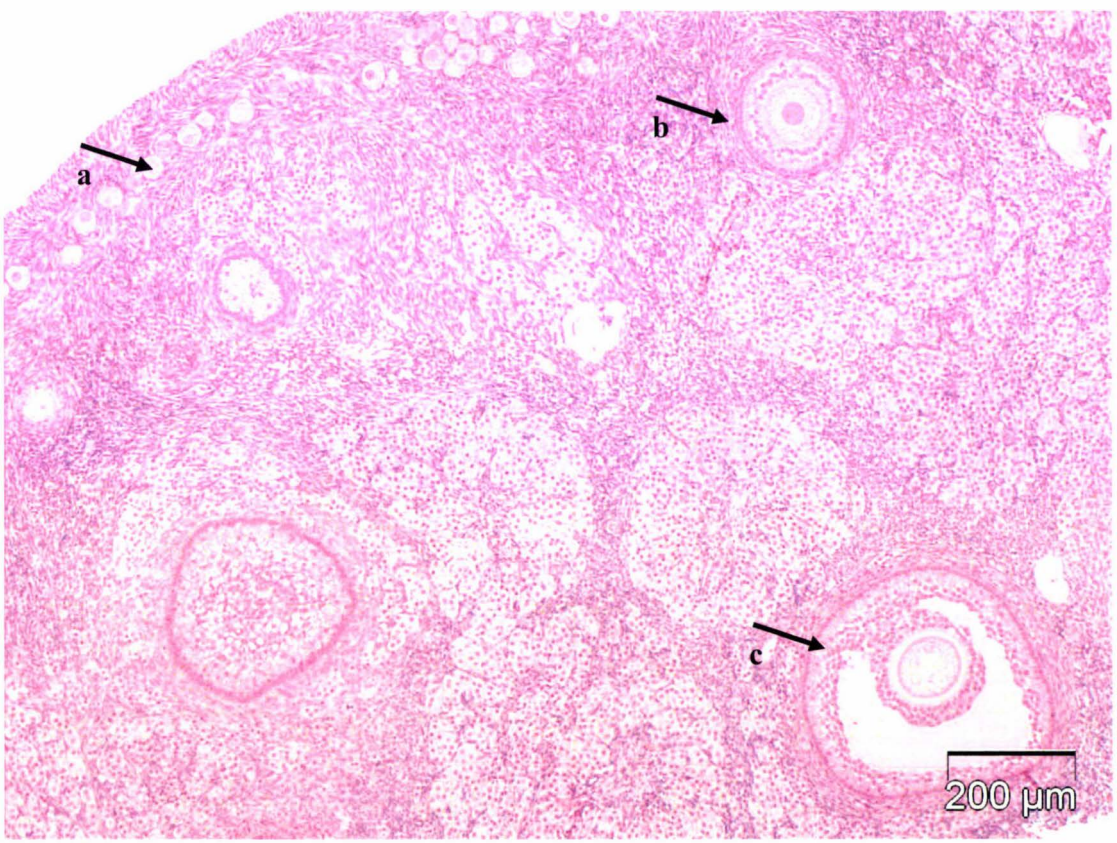


Figure 5.7. Histological section of a cat ovary showing primordial (a), secondary (b) and tertiary (c) follicles (→). Stained with haematoxylin and eosin.

5.2.3.3. *Immunohistochemistry*

A power analysis predicted that a minimum of 19.3% difference in receptor expression could be detected between groups with a power of 95%. The expression of ERα, ERβ and PR was quantified in the uterine endometrium (Figure 5.8), outer endometrium and myometrium, as well as the ovarian cortex and medulla of each cat.

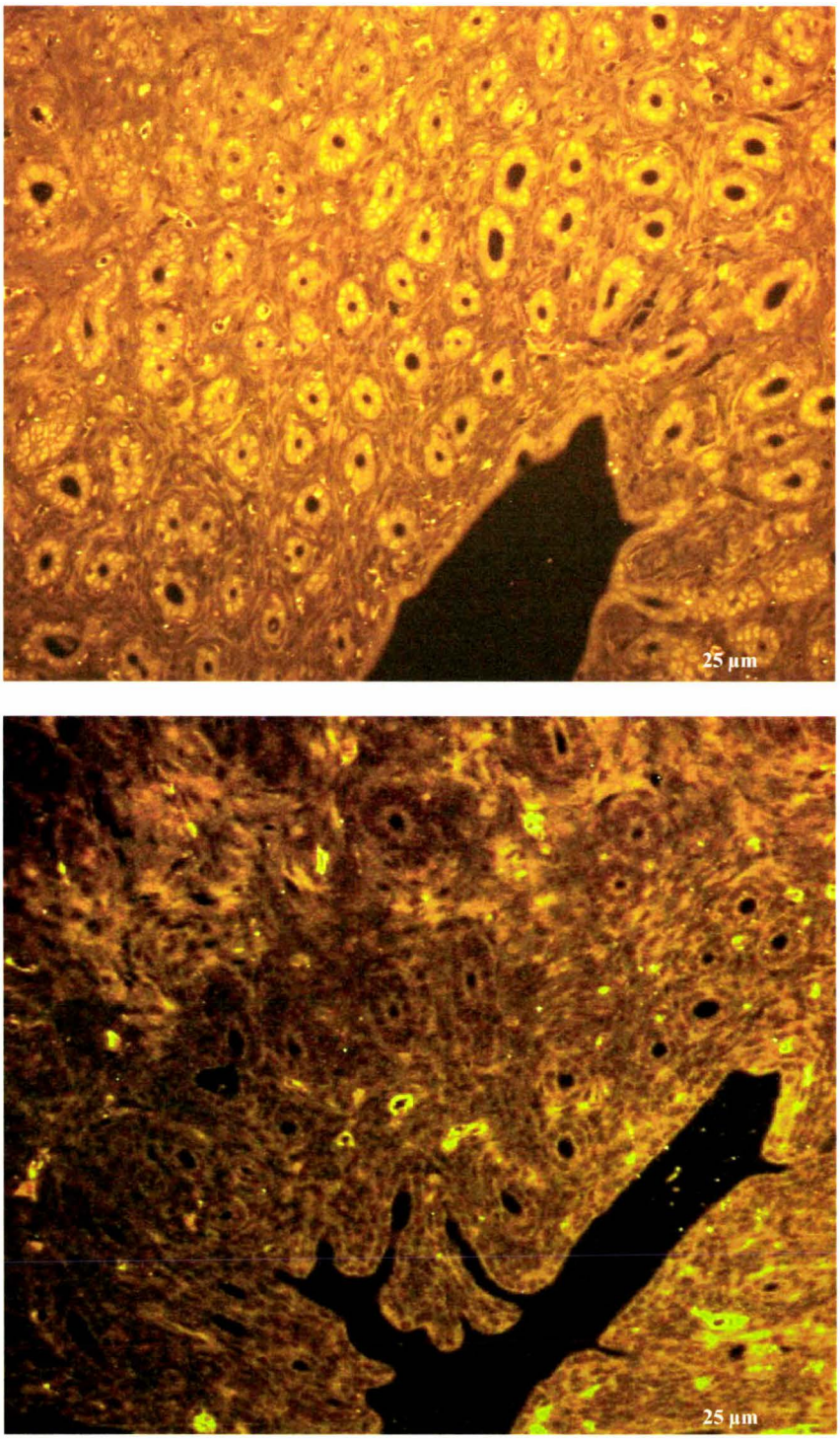


Figure 5.8a. Top: Section of a cat uterine lumen, stained for ERα with monoclonal mouse antibody, IgG1 (NCL-ER-6F11). Bottom: Negative control tissue.

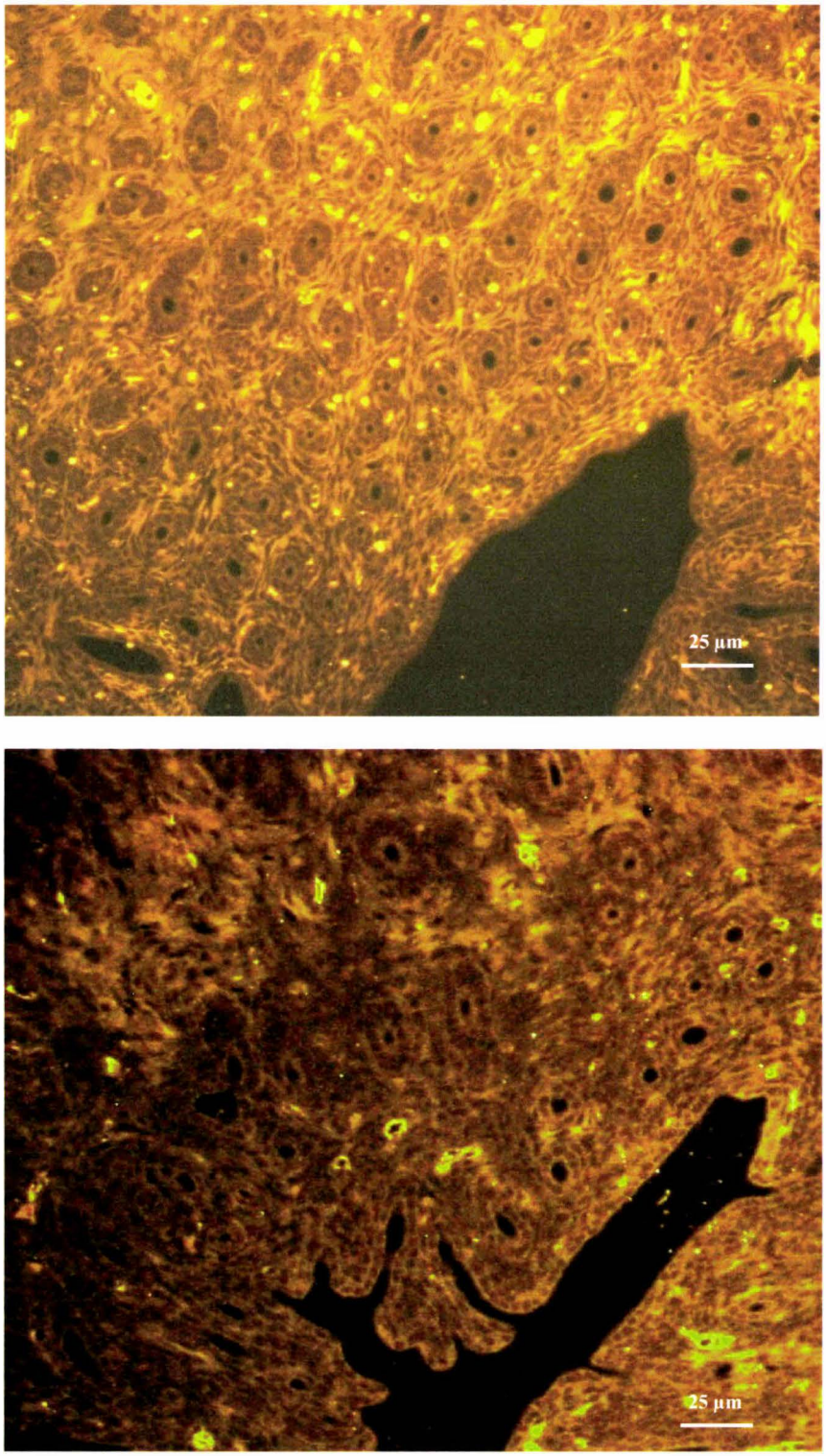


Figure 5.8b. Top: Section of a cat uterine lumen, stained for ERβ with monoclonal mouse antibody, IgG1 (NCL-ER-beta). Bottom: Negative control.

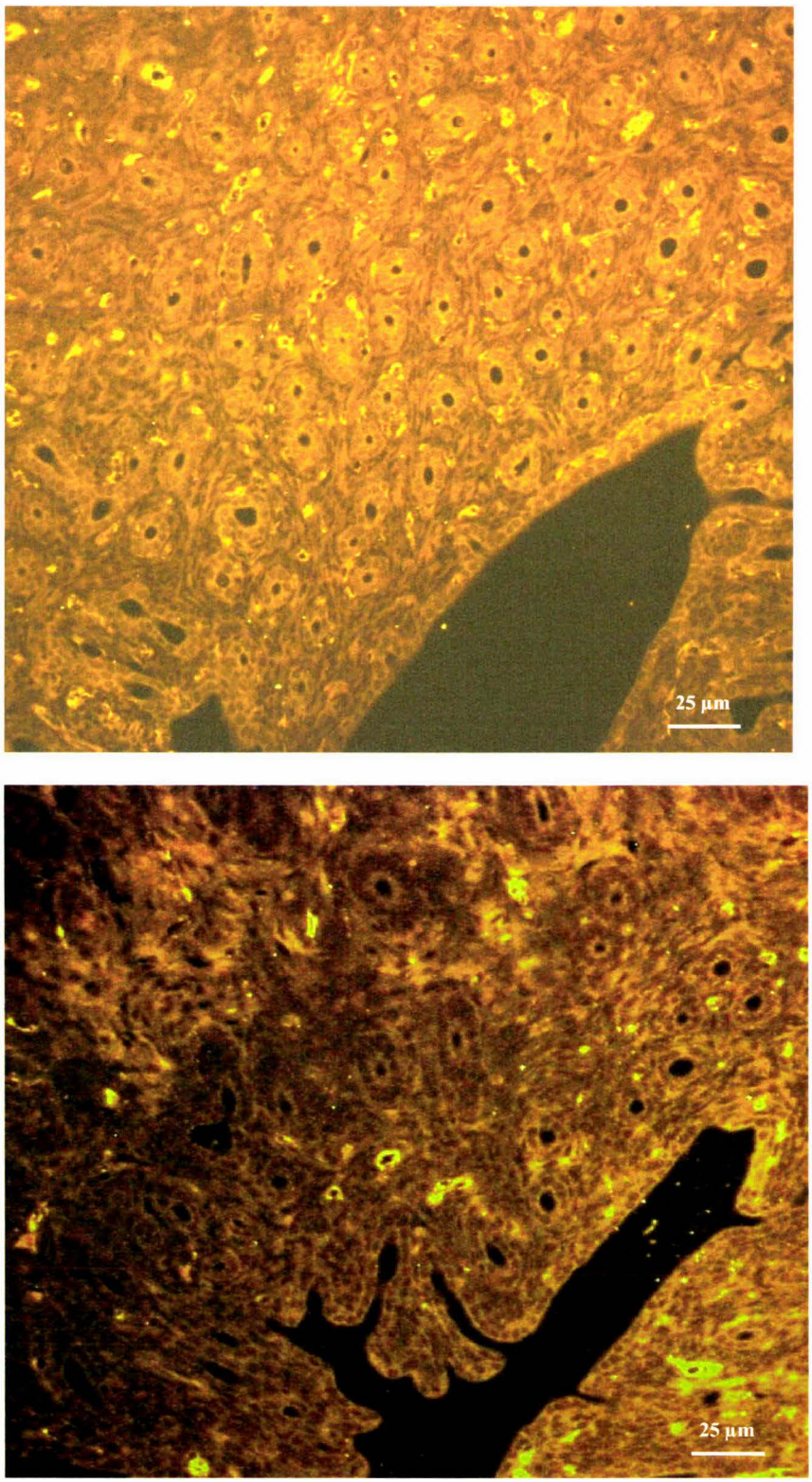


Figure 5.8c. Top: Section of a cat uterine lumen, stained for PR with mouse monoclonal antibody, IgG1 (PR4-12). Bottom: Negative control.

5.2.3.3.a. *Ovarian cortex*

No detectable difference was observed between groups in the intensity of staining in cells containing ERα or ERβ in the ovarian cortex. However, the proportion of cells within the cortex that stained positively for ERα was greater in treatment cats than control cats (median 0.79 *versus* 0.58, respectively, $p < 0.01$). Similarly, a significant difference was observed in ERβ proportional expression between treatment and control cats (0.83 *vs.* 0.57, respectively, $p < 0.01$). Progesterone receptor staining intensity approached significance ($p < 0.10$) for differences between groups, where treatment cats had a slightly lower staining intensity than control cats for this receptor (76.9 ± 5.80 bits, compared to 99.5 ± 6.35 bits, respectively). However, the proportion of cells expressing PR was significantly lower in treatment cats than control cats (0.66 *vs.* 0.75, respectively, $p < 0.01$). This data is summarised in Table 5.4.

	Control Group	Treatment Group
ERα proportion	0.58	0.79
ERα intensity (bits)	74.9	81.0
ERβ proportion	0.57	0.83
ERβ intensity (bits)	98.4	84.8
PR proportion	0.75	0.66
PR intensity (bits)	99.5	76.9

Table 5.4. Summary of mean hormone receptor proportion and intensity in the ovarian cortex of control (N = 8) and treatment cats (N = 4) at the time of ovario-hysterectomy

5.2.3.3.b. *Ovarian medulla*

There was no effect of treatment on the intensity of staining in cells expressing ERα or ERβ in the ovarian medulla. However, treatment cats had a greater proportion of cells expressing both ERα and ERβ in medullary tissue, than the control cats (ERα: 0.94 *vs.* 0.52, respectively, $p < 0.01$, and ERβ: 0.93 *vs.* 0.79, respectively, $p < 0.01$). Dietary isoflavones significantly reduced PR staining intensity in treatment cats (84.8 bits (± 5.64), compared to 111.5 bits (± 1.29) in control cats, $p < 0.05$). However, no significant

difference was detected in the proportion of cells expressing PR, in this tissue type, between groups. This data is summarised in Table 5.5.

	Control Group	Treatment Group
ERα proportion	0.52	0.94
ERα intensity (bits)	88.5	88.0
ERβ proportion	0.79	0.93
ERβ intensity (bits)	109.0	101.9
PR proportion	0.72	0.78
PR intensity (bits)	111.5	84.8

Table 5.5. Summary of mean hormone receptor proportion and intensity in the ovarian medulla of control (N = 8) and treatment cats (N = 4) at the time of ovario-hysterectomy

5.2.3.3.c. *Uterine endometrium*

No significant difference in staining intensity was observed between groups for ERα or ERβ in the uterine endometrium. Treatment cats had a significantly higher proportion of cells expressing ERα (0.95) than control cats (0.91; $p < 0.01$) and a similar result was observed for ERβ (0.88 *vs.* 0.63, respectively; $p < 0.01$). No difference in PR staining intensity was detected between groups although treatment cats had a significantly lower proportion of cells expressing PR, compared to control cats (0.64 *vs.* 0.81, respectively; $p < 0.01$). This data is summarised in Table 5.6.

	Control Group	Treatment Group
ERα proportion	0.91	0.95
ERα intensity (bits)	92.2	121.5
ERβ proportion	0.63	0.88
ERβ intensity (bits)	81.6	108.4
PR proportion	0.81	0.64
PR intensity (bits)	86.6	102.8

Table 5.6. Summary of mean hormone receptor proportion and intensity in the uterine endometrium of control (N = 8) and treatment cats (N = 4) at the time of ovario-hysterectomy

5.2.3.3.d. *Uterine outer endometrium*

No difference in ERα or ERβ staining intensity was detectable between groups in the uterine outer endometrium tissue. Treatment cats had a significantly lower proportion of cells expressing ERα (0.84) than control cats (0.91; $p < 0.01$), but the opposite relationship was found for ERβ (0.95 *vs.* 0.79 for treatment and control groups, respectively; $p < 0.01$). The proportion of cells in treatment cat sections expressing PR (0.88) was significantly higher than that of control cats (0.76; $p < 0.01$). This data is summarised in Table 5.7.

	Control Group	Treatment Group
ERα proportion	0.91	0.84
ERα intensity (bits)	90.1	102.9
ERβ proportion	0.76	0.95
ERβ intensity (bits)	83.6	93.8
PR proportion	0.73	0.88
PR intensity (bits)	81.6	85.0

Table 5.7. Summary of mean hormone receptor proportion and intensity in the outer uterine endometrium of control (N = 8) and treatment cats (N = 4) at the time of ovario-hysterectomy

5.2.3.3.e. *Myometrium*

No significant differences were detected between the staining intensity of ERα, ERβ or PR-stained cells in the treatment and control groups. However, the proportion of cells expressing ERα in the treatment group (0.32) was significantly lower than that of the control cats (0.60; $p < 0.01$). No difference was detected between groups in the proportion of cells expressing ERβ, although a trend towards increased expression by treatment cats was detected ($p < 0.10$). Dietary treatment did not significantly affect the proportion of PR expression. This data is summarised in Table 5.8.

	Control Group	Treatment Group
ERα proportion	0.40	0.68
ERα intensity (bits)	83.0	94.4
ERβ proportion	0.50	0.42
ERβ intensity (bits)	86.3	87.6
PR proportion	0.38	0.36
PR intensity (bits)	89.7	84.6

Table 5.8. Summary of mean hormone receptor proportion and intensity in the uterine myometrium of control (N = 8) and treatment cats (N = 4) at the time of ovario-hysterectomy

5.2.3.3.f. *Receptor distribution within tissues*

Using only data from control cat tissues to determine normal receptor distribution within tissues, the expression of ERα was significantly greater in the endometrium than the myometrium (38.2% compared to 30.9% of the total tissue expression, respectively; $p < 0.01$). No difference was detectable between expression in the outer endometrium, compared to the endometrium, or to the myometrium. A trend was detected towards greater expression of ERα in the ovarian medulla, compared to the cortex (55.5% *vs.* 44.5%, respectively; $p < 0.10$).

Expression of ERβ did not differ significantly between control cat endometrium, outer endometrium or myometrium. Likewise, no difference was detectable between the relative expression of ERβ in the ovarian tissue regions of control cats.

No significant differences in PR expression were detectable between any regions of uterine or ovarian tissue in control cats. However, a trend was detected towards higher PR expression in the endometrium, compared to the outer endometrium (34.4% and 31.5%, respectively, $p < 0.10$). The ovarian medulla also had slightly higher relative PR expression (53.8%) than the cortex (46.2%; $p < 0.10$).

As a proportion of the total positively-stained receptors within the uterus of each cat (combining the endometrium, outer endometrium and myometrium), receptors appeared to be relatively uniformly distributed among each region of this tissue. The same distribution was observed in the ovary (combining the medulla and cortex). No significant differences were detected in the regional distribution of ERα, ERβ or PR in the uterus or ovary between control and treatment cats. In addition, no between-group differences were detected in the ratio of ERα to ERβ in any tissue type.

5.2.4. Discussion

Dietary isoflavones did not elicit any change in the wet weight or gross morphology of the reproductive tract in domestic cats, when provided as part of a dietary matrix (300 μg isoflavones/g dry matter) from weaning until over 400 days of age. This is in agreement with previous reports of a lack of uterotrophic effects in other species (Penotti *et al.* 2003; Eason *et al.* 2005; Diel *et al.* 2006; Wood *et al.* 2006ab).

In contrast, isoflavone-induced histological changes and modulation of sex steroid receptor expression were apparent in this study. These findings suggest that a change in the wet weight of reproductive organs may not be a sufficiently sensitive marker for the biological activity of isoflavones in the cat. Histological, cytological and molecular changes, in the absence of concomitant uterotrophic effects, have also been reported for other species following isoflavone exposure (Diel *et al.* 2002; Jefferson *et al.* 2002b; Selvaraj *et al.* 2004; Hertrampf *et al.* 2005).

Dietary isoflavone exposure resulted in an increased luminal epithelial cell height (LEH) of treatment cats, which is in agreement with findings in a number of other non-felid studies (Gallo *et al.* 1999; Brown and Lamartiniere 2000; Cotroneo *et al.* 2001; Diel *et al.* 2002; Jefferson *et al.* 2002b; Ford *et al.* 2006; McClain *et al.* 2006b), and makes growth

stimulatory effects feasible. The predominance of more columnar cells in the glandular epithelium of the uterine horns of treated cats is also indicative of cellular hypertrophy.

The isoflavone-induced increase in LEH reported here was not as dramatic as reported following exogenous oestrogen administration in cats (Bareither and Verhage 1980) or genistein exposure in rats (Cotroneo *et al.* 2005). This may be due to interactions with endogenous circulating oestrogens, since cats in the present study were not gonadectomised prior to isoflavone exposure. The increase in LEH observed in the current study following isoflavone treatment (7.62 μm *vs.* 6.50 μm) also did not approach the degree of increase that is observed during a normal feline oestrous cycle (Chatdarong *et al.* 2005). During a normal oestrous cycle LEH is highest during the follicular and luteal phases (14.44 μm and 14.31 μm, respectively) and significantly lower during the inactive phase (5.93 μm; Chatdarong *et al.* 2005). Hence, isoflavones may mimic endogenous oestrogen in their capacity to increase LEH, but the overall effect is much smaller than the changes that typically occur during the normal oestrous cycle of the domestic cat. The cellular changes observed in the current study may therefore not have significant consequences for fertility or fecundity. However, exposure during critical developmental stages (*in utero* and/or prior to weaning), or for a prolonged period, may produce divergent results.

Previous reports of the low relative biological potential of isoflavones, compared to endogenous oestrogens (Casanova *et al.* 1999; Branham *et al.* 2002; Lehmann *et al.* 2005), support the findings of the present study. It is likely that the poor oral availability of genistein and daidzein in the domestic cat (Section 3.3), as well as factors such as the dose, age at exposure and duration of exposure were important in reducing the observed effect of isoflavones in this regards.

Mixed agonistic and antagonistic effects have been reported in ovariectomised (OVX) rats exposed to varying doses of combined exogenous oestradiol and genistein, including

a genistein-related dampening of the oestradiol-induced increase in LEH (Diel *et al.* 2006). It is therefore possible that the endogenous oestrogen of the intact cats in the present study exerted an interactive or influential effect on isoflavone activity. Collection of histological tracts of cats in the oestrus phase, following isoflavone-exposure, as well as from OVX isoflavone-exposed cats, would be necessary to determine the extent of this interaction.

A lack of isoflavone-induced effect on LEH has been reported (Mäkelä *et al.* 1995; Tansey *et al.* 1998; Hughes *et al.* 2004; Naciff *et al.* 2004; Eason *et al.* 2005; Nikaido *et al.* 2005; Wood *et al.* 2006a). However, the majority of studies did not standardise animal measurement according to the stage of oestrous, despite the natural variation in histological parameters present during a typical oestrous cycle. By collecting tissue from animals in the same stage of oestrous (this study), the power to detect changes was increased. The accuracy with which inter-oestrous was determined by vaginal cytology in this study was supported by the finding that the LEH of all cats was within the normal cell height for cats in the inactive phase of the oestrous cycle (Chatdarong *et al.* 2005). The lack of significant glandular secretion observed in histological sections further supports an inactive state.

Folliculogenesis appeared to be unaffected by isoflavones in the current study, with follicles at each stage of development found in comparable numbers in the ovaries of both treated and untreated cats. This is in contrast to studies in rodents, which report an alteration of follicle size, distribution and development, following isoflavone exposure (Awoniyi *et al.* 1998; Gallo *et al.* 1999; Jefferson *et al.* 2002a; Masutomi *et al.* 2003). However, domestic cat folliculogenesis exhibits some unique characteristics which complicate the extrapolation of potential isoflavone effects from rodent models. Multi-oocyte follicle (MOF) formation has been associated with neonatal isoflavone exposure in rodents, and is thought to be mediated by ERβ (Jefferson *et al.* 2002a and 2006). However, MOF's occur frequently in untreated juvenile cats (Bristol-Gould and Woodruff 2006). Additionally, deficiencies in oocyte-secreted growth factors, insulin

growth factor signalling pathways and over-expression of the inhibin-α gene are also known to induce MOFs in other species (Jefferson *et al.* 2002a). Hence, one or any of these factors may be naturally-occurring in cats, so that MOF's may not be a reliable indicator of ovarian isoflavone activity in this species. Furthermore, despite an upregulation of ERβ, detected in this study (discussed below), which has previously been linked to follicular developmental changes, no changes were detected in follicle development in isoflavone-exposed cats in this study.

The lack of an isoflavone effect on folliculogenesis in cats may instead be related to the timing of exposure. Primary oocyte formation is completed in the cat shortly after birth (Bristol-Gould and Woodruff 2006), so it is possible that folliculogenesis was unaffected by post-weaning exposure to significant concentrations of isoflavones. Therefore, additional studies are required to investigate the impact of neonatal and/or gestational isoflavone exposure in cats.

Suppurative endometritis (pyometra) is not common in cats, and is generally only reported in cats older than 8 years of age (Agudelo 2005). The incidence of younger cats exhibiting this condition is typically restricted to animals treated with progestagens or oestrogens for contraceptive or abortive means (Agudelo 2005). Pyometra is a progesterone-induced condition, but is also influenced by oestrogen since this hormone acts to increase PR, dilate the cervix (which permits bacterial ascent from the vagina), and influences endometrial changes (Agudelo 2005). Furthermore, pyometra has also been linked to ovulation, and the presence of ovarian corpora lutea (Agudelo 2005).

The finding of pyometra in a treatment cat may be associated with dietary isoflavone intake. Given the association of isoflavones with apparent spontaneous ovulation (Section 5.1.3.6.), and the fact that this cat was housed and cared for under identical conditions as cats in the control group, the possibility exists that the observed pyometra was not a random event. The affected cat was under one year of age at the time of

diagnosis, and as such, this case can be considered unusual. Furthermore, the absence of pyometra in any of the control group animals, which were genetically related to the affected cat, is further suggestive of an isoflavone-related influence. Isoflavones did not consistently up-regulate PR expression in this study, although it is unknown if they were acting as oestrogen mimics and affecting other functions (e.g. cervical dilatation or modulation of endometrial activity) in order to induce a pyometra in this case. It is also feasible that the observed increase in apparent spontaneous ovulation, and/or increased demonstration of sexual behaviour (Section 5.1.3.6. and 5.1.3.8.) were factors in the aetiology of this condition. Unfortunately the equol-producer status of cats in this study was not determined. It is possible that the affected cat reported here was an equol-producer where other cats were not and that the production of this potent isoflavone contributed to the observed pyometra. Given the isolated incidence in this study, further investigation with a larger population of cats would be necessary to confirm the link between pyometra and isoflavone exposure.

A potential problem with this study was the use of a maternal diet that contained detectable isoflavone levels. However, none of the diets assayed to be devoid of isoflavones (Section 2.1) met the nutritional requirements for gestation and lactation in domestic cats (NRC 2006). As such, all kittens were exposed to isoflavones *via* gestation and lactation, prior to the onset of this study. The maternal diet in this study did, however, provide isoflavones in significantly lower concentrations (0.56 mg – 0.84 mg/kg BW) than the minimum effective dose reported for rodents (> 4 mg/kg BW) exposed *via* these routes (Kang *et al.* 2002). Furthermore, pre-weaning exposure was standardised across groups. Hence, *in utero* and/or lactational exposure of kittens is considered minimal and unlikely to have influenced the parameters assessed in this study. Nevertheless, it is still feasible that pre-weaning exposure in both groups may have reduced the power of the study to detect changes associated with post-weaning exposure.

Immunohistochemical analysis of ovarian and uterine tissues revealed some significant changes in isoflavone-treated cats, compared to controls. Generally, the staining intensity

of cells positive for the ERα, ERβ and PR was unaffected by isoflavones. This suggests that, where present, the receptor protein content of cells did not change in response to isoflavone exposure. However, the proportion of cells staining positive for these receptors within a given tissue was altered in treatment compared to control animals. This indicates the potential for an overall effect on receptor expression. The up-regulation of the number of cells containing ERα and ERβ in the ovarian cortex, medulla and uterine endometrium observed here agrees with findings in other species (Patisaul *et al.* 2001; Jefferson *et al.* 2002a; Laurenzana *et al.* 2002; Lee *et al.* 2004; Chrzan and Bradford 2007). Exogenous oestradiol has also been shown to increase ER staining in OVX cats (West *et al.* 1976; Li *et al.* 1992). Likewise, expression of ERα is modulated during normal oestrous cycles in cats, being up-regulated during pro-oestrus and oestrus (West *et al.* 1977). Since tissue collection was performed during apparent inter-oestrus periods in all cats, it appears that isoflavones were acting as oestrogen-mimics in this study. However, other studies report a down-regulation of these receptors in an oestrogen-antagonistic manner, perhaps indicating alternative mechanisms of activity in other species and/or tissues (Ren *et al.* 2001; Wang *et al.* 2005; Diel *et al.* 2006; Takashima-Sasaki *et al.* 2006). Differences in dose, duration of exposure, as well as interspecific and tissue differences are likely to play a role in modulating the observed divergent results.

Badger *et al.* (2001) failed to detect uterotrophic effects in rats that exhibited suppression of circulating oestrogens. It has been suggested that since uterotrophy is primarily an ERα-mediated effect, an altered circulating hormone profile may be elicited through other pathways (Badger *et al.* 2001). However, up-regulation of ERα observed in this study, in conjunction with a lack of effect on uterine wet weight, suggests that the degree of upregulation of ERα was insufficient to elicit significant uterotrophic changes. However, given the increase in LEH in cats exposed to isoflavones, it is feasible that continued exposure may have brought about detectable uterotrophic differences between groups.

Oestrogen is an anti-atretic factor in the ovary and facilitates ovulation (Jefferson *et al.* 2002a). The up-regulation of ER expression observed in the ovaries of isoflavone-exposed cats may consequentially have promoted ovulation capacity (Jefferson *et al.* 2002a) and could partially explain the findings of increased spontaneous ovulation incidence (see Section 5.1.3.6). On the other hand, upregulation of the ER in other components of the neuroendocrine system of cats may also have resulted in the oestrogenisation of the hypothalamus-pituitary axis (Jefferson *et al.* 2002a). This, in turn, may have rendered the ovaries more sensitive to low concentrations of gonadotrophins released following non-mating stimuli, or alternatively elicited greater luteinising hormone release from the pituitary gland. Either of these scenarios may have been sufficient to induce spontaneous ovulation.

Endogenous sex steroids regulate their own receptor distribution in the cat uterus, and the suppression of ER during pregnancy or progesterone administration has been associated with reduced oviduct function, but enhanced growth and secretory processes in uterine tissues (West *et al.* 1976 and 1977; Li *et al.* 1992). The coordinated, cyclic and counteracting functions of oestrogen and progesterone are hence critical for normal functioning of female reproductive organs (Diel *et al.* 2000; Wang *et al.* 2005). Modulation of ER expression in the cat uterus may therefore have knock-on effects for tissue responses to endogenous oestrogens, as well as implications for the expression of other oestrogen-responsive genes, such as mitogenic substances, inflammatory cytokines, tumour-associated antigens and mediators of bone turnover (Jefferson *et al.* 2002a; Lee *et al.* 2004; Chrzan and Bradford 2007).

The novel data presented here for ERβ indicates that the oestrogen receptors, ERα and ERβ, are relatively uniformly distributed within normal (control) feline ovarian tissue. However, differences were detected in uterine tissue. Generally, the expression of both receptors was lowest in the myometrium, but the difference was less apparent for ERβ between uterine tissues, than it was for ERα. If isoflavones were exerting their activity primarily through the ERα, this modest difference in distribution may partially explain

the heightened response to isoflavone treatment observed in the endometrium (e.g. increased LEH and changes in receptor expression) compared to other tissues.

Elucidation of the pathway(s) by which isoflavones exert their influence over sex steroid receptors was not a focus of this study, but previous reports suggest multi-factorial mechanisms are likely. Oestrogens are involved in the regulation of growth and differentiation of many tissues (Jefferson *et al.* 2002a) and oestrogenic compounds possessing transcriptional activities for the ER (*via* direct or indirect DNA-binding) affect a diverse range of response elements and oestrogen-responsive genes (Lee *et al.* 2004). Furthermore, interaction with the ER is not the only pathway available to induce oestrogen-like effects in the reproductive tract (Diel *et al.* 2000). Direct or indirect action on the pituitary gland, non-genomic activation, or cross-talk with certain signal transduction cascades (e.g. intracellular calcium levels and tyrosine kinase inhibition), or binding to other steroid receptors and/or steroid hormones may explain the divergent molecular activities of isoflavones (Diel *et al.* 2000; Jefferson *et al.* 2002a ; Lee *et al.* 2004; Ford *et al.* 2006).

In addition, isoflavones may bind to the carrier proteins of endogenous sex steroids, and reduce their transport around the body, or may interact directly with hormones (Hollander 1997). This in turn may result in negative feedback and modulation of hormone receptor expression. However, it is unclear whether hormone concentrations are directly or indirectly affected and likewise, whether receptor expression is a primary or secondary effect of isoflavone exposure. Sex steroid plasma concentrations were not measured in this study, although the apparent lack of effect on vaginal cytology indicates that isoflavones, at the dose utilised, were unable to modulate hormone exposure of the vagina, and perhaps other components of the reproductive tract. Hence, the modulation of receptor expression is unlikely to be a result of aberrant endogenous hormone exposure, suggesting that isoflavone activity is more direct, acting *via* ligand-binding of the ER, and/or non-transcriptional interference.

Differences in isoflavone-induced effects were tissue- and ER-specific. Unlike the findings in the endometrium of the uterus, the number of cells expressing ERα was down-regulated by isoflavones in the outer endometrium and myometrium, suggesting an anti-oestrogenic role for isoflavones in these tissues. However ERβ expression was consistently up-regulated in both the endometrium and outer endometrium, but unaffected in the myometrium. Overall, the myometrium appeared to be the least sensitive to isoflavone-induced modulation of receptor content. This agrees with previous findings in cats demonstrating mild hyperplasia, where a relative lack of effect was observed in ERα and PR expression in the myometrium, but endometrial cell expression was modulated under the hyperplastic condition (Misirlioglu *et al.* 2006).

An ERα down-regulating effect has previously been observed following exogenous treatment with progesterone in cats (West *et al.* 1976; Brenner *et al.* 1979; Verhage and Jaffe 1986; Li *et al.* 1992), as well being associated with the suppression of oestradiol-induced changes in ER expression (West *et al.* 1976; Li *et al.* 1992). Hence, isoflavones in this study are thought to have been acting as oestrogen-mimics on the expression of ERα in the endometrium, but in an opposing manner in other tissue regions.

The lower expression of ERα in the myometrium may offer an explanation for reduced responsiveness to isoflavones detected in this tissue type. The consequences of exogenous hormone exposure on the ERβ in the cat have not been studied but the results of this study suggest that isoflavone activity against this receptor is less variable in its direction. Isoflavones have a greater affinity for ERβ over ERα, and contrasting responses have been observed according to the predominant form of ER expressed within a tissue (Barnes *et al.* 2000; An *et al.* 2001; Bennetau-Pelissero *et al.* 2001; Ford *et al.* 2006; Chrzan and Bradford 2007). Genistein, in particular, has a high affinity for ERβ (Branham *et al.* 2002; Hwang *et al.* 2006), with some reports suggesting it is nearly equivalent to oestradiol in its relative binding affinity (Nikov *et al.* 1997; Kuiper *et al.* 1998). This contrasts sharply with its affinity for ERα (3 - 4%) (Kuiper *et al.* 1998; Hwang *et al.* 2006). Furthermore, the conversion of daidzein to *O*-DMA aglycone

(detected in the plasma of a cheetah in Section 4.1.3.2.) increases its binding affinity for ERβ substantially, to levels equivalent to oestradiol (Kinjo *et al.* 2004). The greater affinity for ERβ may partially explain the more consistent up-regulation of ERβ.

However, unlike isoflavones, oestradiol binds both ERα and ERβ equally. Therefore, normal circulating concentrations of endogenous oestradiol may suppress isoflavone ER-binding, or restrict it to tissues of high ER expression; in particular those with significant ERβ content. As a result of this potential varying receptor-binding across tissue types, isoflavones may be exerting biphasic effects according to the relative degree of receptor distribution. Consequently, this may result in their primarily oestrogen-like effect in tissue types where competition for receptors with endogenous oestrogen is lowered (e.g. endometrium). However, in tissues with lower ERα expression (e.g. myometrium), isoflavone binding to ERβ receptor may be enhanced and their anti-oestrogen activities may be more pronounced. Confirmation of this hypothesis is beyond the scope of this study.

Proportional expression of the PR was typically down-regulated in isoflavone-treated cat uterine and ovarian tissues. The exceptions to this were the apparent upregulation of PR expression in the outer endometrium, and lack of effect in the myometrium. In contrast to the findings of this study, genistein is known to increase PR expression in other species (Hughes *et al.* 2004; Wang *et al.* 2005; Ford *et al.* 2006). Since exogenous oestradiol elicits an increase in PR staining in OVX cats (Li *et al.* 1992) and other species (Ford *et al.* 2006), the findings of this study suggest that isoflavones were exerting anti-oestrogenic effects. Progesterone has a similar suppressive effect on its own receptor in cats (Verhage and Jaffe 1986), and functions to oppose oestrogenic activity, further supporting an antagonistic function for isoflavones towards this receptor.

Regulation of uterine proliferation, implantation and maintenance of pregnancy, is mediated by the PR (Hughes *et al.* 2004). Hence, PR suppression in the cat suggests a

deficient or aberrant uterine tissue response to hormonal stimuli may be observed (Hughes *et al.* 2004). This effect is most likely to become evident during implantation, pregnancy or in the susceptibility to cancer (Hughes *et al.* 2004). Therefore, future trials investigating conception, implantation and maintenance of pregnancy, in the isoflavone-exposed cat are warranted.

5.3. Conclusions

- Chronic exposure of domestic cats, to a diet containing 300 μg isoflavones/g DM (approximately 5 mg/kg BW), comprising equal parts of genistein and daidzein, was ineffective in modulating oestrous cycle characteristics, or increasing uterine or ovarian wet weight.

- Vaginal cytology was apparently unaffected by dietary isoflavones, which is consistent with previous findings, where a dose of 100 mg/kg BW was necessary to demonstrate oestrogen-like changes in vaginal cytology in the cat (Cave *et al.* 2007c).

- However, dietary isoflavones were successful in increasing the incidence of apparent spontaneous ovulation, and modulating histological and molecular parameters in this species.

- Although uterotrophic studies are considered to be the "gold-standard" for detection of oestrogenic effects by isoflavones in other species, this parameter was insensitive and should not be utilised as a marker for isoflavone-related biological activity in the cat.

- The sex steroid receptors, ERβ and PR, are generally uniformly distributed throughout the uterus and ovary. However, expression of ERα was typically higher in the uterine endometrium. Histological changes were also more apparent in response to isoflavone exposure in the endometrium, suggesting a role for ERα-mediated pathways in isoflavone activity.

- Tissue-specific and ER-specific differences in isoflavone activity were apparent from this study. The endometrial region of uterine tissue demonstrated ERα receptor up-regulation following isoflavone exposure. However, other uterine tissue types exhibited a down-regulation of ERα expression. Changes in expression of ERβ and PR was more consistent across tissue regions, with ERβ being up-regulated and PR being down-regulated in response to isoflavone exposure.

- The monitoring of multiple endpoints in this study, including cytological, histological and molecular markers, appeared to have increased its power to detect changes in the domestic cat reproductive tract.

- A trend towards an increased level of sexual behaviour demonstration was also observed in the isoflavone-treated group. This requires further testing as behavioural data was collected opportunistically.

- Further testing of the influence of isoflavones in the feline reproductive tract is required. Future trials should include larger sample sizes, as well as validation of the determination of the stage of oestrous, spontaneous ovulation and sexual behaviour with concurrent circulating hormone concentrations.

- Controlled mating, conception, gestation and kitten-rearing studies are required to determine the long-term effect of isoflavones on feline reproductive capacity and health. *In utero* and/or neonatal exposure may also confer divergent effects and warrants investigation.

CHAPTER SIX

The Effect of Long-term Dietary Intake of Genistein and Daidzein on Hepatic Histopathology, Enzyme and Bile Acid Concentrations in Domestic Cats (*Felis catus*)

6.0. Introduction

The second major hypothesis of Setchell *et al.* (1987ab) regarding the influence of dietary isoflavones in the physiology of captive cheetahs was that isoflavones may be associated with impaired hepatic function and/or diseases such as veno-occlusive disease (VOD). Indeed, the liver is also a target of oestrogen activity (Diel *et al.* 2002) and isoflavones have been shown to exert a variety of effects on hepatic activities (see Section 1.6). However, in contrast to the hypothesis of Setchell *et al.* (1987ab), isoflavones have been reported to be protective against various hepatic insults (Lee *et al.* 1995; Kang *et al.* 2001; Liu *et al.* 2002; Kuzu *et al.* 2007; Wong *et al.* 2007).

The previous chapter demonstrated that isoflavone exposure resulted in detectable histological and molecular changes in the reproductive tract of the domestic cat (see Section 5.2.4.). It is, therefore, feasible that changes may occur elsewhere in feline physiology. Although liver disease in captive cheetahs has been tentatively associated with dietary isoflavone consumption (Setchell *et al.* 1987ab), no controlled study has been conducted to determine the existence and/or nature of such an association. The aim of this study was therefore to determine if long term consumption of genistein and daidzein had any detectable effect on hepatic histology or biochemical parameters in domestic cats. The form of isoflavone administration utilised here was identical to that used in Section 3.3, where the availability of genistein was calculated to be 54%. The

availability of daidzein was calculated to be 29%. The domestic cat is used as a model species for the captive cheetah.

6.1. Experiment One: Hepatic Biochemistry Following Chronic Ingestion of Genistein and Daidzein

6.1.1. Aim

Hepatic enzyme concentrations provide an indication of hepatocyte health and biliary secretion. The aim of this study was to evaluate the effect of chronic ingestion of genistein and daidzein on hepatic enzyme and bile acid concentrations in domestic cats. Individual cats were assessed for hepatic enzyme and bile acid production, before and after the removal of isoflavones from their diet. These same parameters were compared between previously exposed cats and unexposed control animals.

6.1.2. Materials and Methods

6.1.2.1. *Animals*

Eleven female short-haired domestic cats were utilised in this study. Cats assigned to the treatment group (n = 4) had been exposed to the dietary isoflavones, genistein and daidzein, since weaning. The same cats were used in this study as in Section 5.2 with the exception of one further death following ovario-hysterectomy. The death of this cat was unrelated to the ovario-hysterectomy procedure and was not considered to have interfered with the data collected prior to ovario-hysterectomy. Cats in the control group (n = 7) consumed the same base diet as the treatment group, except that isoflavones were not added to this diet. The average age at initial blood collection was 428 (± 25.75) days. One female in the treatment group developed a pyometra, which necessitated early surgical removal of the reproductive tract at age 267 days (see section 5.2.2.2). Blood

collection from this cat was obtained at this time. Removal of this female's age from the calculation of the average gives a mean age of 485 (± 19.17) days. Cats had consumed the trial diets for an average of 394 (± 25.73) days at the time of blood collection.

Dietary isoflavone administration and husbandry conditions are described in Section 5.1. Ethical approval for this trial was obtained from the Massey University Animal Ethics Committee (2006).

6.1.2.2. *Serum collection*

Cats were fasted overnight, prior to an initial (2 ml) blood sample being collected by venipuncture of the jugular vein. The cats were then offered a meal of base diet, and a second (1 ml) blood sample withdrawn by venipuncture 2 h after ingestion of this meal. Blood was transferred into vacutainers and centrifuged to collect serum. This blood sampling regime was conducted prior to the collection of reproductive tissues (described in Section 5.2.2.2).

Following collection of initial blood samples, the diet of the treatment group was changed to that of the control diet (devoid of isoflavones), while the cats in the control group continued to be maintained on the control diet. Forty days following this dietary change, a second pair of blood samples were collected and analysed, as described above.

6.1.2.3. *Serum analysis*

Serum, from the pre-prandial blood sample, was analysed for alkaline phosphate (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT) and bile acids (Gribbles Pathology Ltd., Palmerston North, NZ). Serum from the post-prandial blood sample was analysed for bile acid concentration only

(Gribbles Pathology Ltd., Palmerston North, NZ). Serum was analysed within 48 hours of collection.

6.1.2.4. *Statistical analyses*

Changes in the serum concentrations of enzymes over time were calculated for each cat, and groups were tested for significant differences in any temporal changes. Differences in biochemical parameters at the first sampling time (prior to removal of isoflavones from the treatment group's diet), were also tested between groups. Residual data was tested for normality using the Anderson-Darling test. Normally distributed data was tested for difference between groups using a one-way ANOVA. Differences between groups were tested for significance using the Mann-Whitney test when data was not normally distributed (change in GGT over time, initial ALP and initial ALT). Where data was not normally distributed, the median is reported instead of the mean (Glantz 2005). All statistical analyses were performed with Minitab software (version 15, Minitab Inc., PA, USA) and the level of significance set at $p < 0.05$. Data are expressed as mean (± SEM), unless otherwise stated.

6.1.3. Results

No significant differences ($p > 0.05$) were detected in any biochemical parameters (ALP, AST, ALT, GGT, fasted or fed bile acids) within the first sampling phase, prior to isoflavone removal from the diet of the treatment cats (Table 6.1). Changes in these parameters, within each cat (before and after dietary change) as well as bile acid response to a meal did not differ between groups ($p > 0.05$) (Table 6.2). All parameters were within normal reference ranges for domestic cats, at all time points.

A power analysis of the data indicated that a minimum difference of 59.6% in biochemical parameters could be detected with a power of 95%. This minimum difference was reduced to 46.1% with a power of 80%.

	ALP* (U/L)	AST (U/L)	ALT* (U/L)	GGT* (U/L)	Pre-prandial bile acids (μmol/L)	Post-prandial bile acids (μmol/L)	Bile acid response to a meal (U/L)
Control	29.0	25.7 (± 3.76)	43.0	1.00	0.48 (± 0.08)	1.73 (± 0.29)	1.26 (± 0.31)
Treatment	56.0	27.8 (± 7.53)	44.5	0.50	0.48 (± 0.22)	2.45 (± 0.47)	1.98 (± 0.47)

Table 6.1. Mean (± SEM) hepatic biochemistry parameters following a 394 day (± 25.73) period of dietary isoflavone exposure in the treatment group. *Median data is reported since data for these parameters were not normally distributed.

	ALP (U/L)	AST (U/L)	ALT (U/L)	GGT* (U/L)	Pre-prandial bile acids (μmol/L)	Post Prandial Bile Acids (μmol/L)	Bile acid response to a meal (U/L)
Control	4.00 (± 10.3)	-5.43 (± 10.3)	37.3 (± 2.96)	2.00	1.34 (± 0.65)	0.63 (± 0.74)	-0.70 (± 1.13)
Treatment	-8.25 (± 9.38)	2.75 (± 9.36)	34.5 (±14.5)	4.5	0.03 (± 0.08)	0.35 (± 0.03)	0.40 (± 0.37)

Table 6.2. Mean (±SEM) change in hepatic biochemistry parameters after a 40 days period following the removal of isoflavones from the treatment group cats (no dietary change in the control cats). *Median data is reported for GGT change in time since data for this parameter was not normally distributed.

6.1.4. Discussion

Changes in hepatic biochemistry parameters were generally similar between control and treatment cats. However, the results of the power analysis suggests that the study was poorly designed to detect subtle differences and that only differences of over 55% would be considered significant. Interestingly, the control cats showed changes in some

parameters following the 40-day period, despite no change in their diet. The intra-individual variation in some of the parameters is likely to explain the majority of these changes, and may indicate that the sample size utilised here lacked statistical power to detect any isoflavone-induced changes. However, the cause of these temporal changes may also be analytical as the serum was analysed immediately following collection and therefore the pre- and post-dietary manipulation samples were analysed in separate batches.

The consistent increase in ALT and GGT production, observed in both control and treatment groups, may reflect altered metabolism or hepatic activity as a consequence of removal of the reproductive tract, which occurred prior to the time that isoflavones were removed from the diet of treatment cats. Induction of ALT and GGT is known to involve hormonal action, or to occur as a consequence of muscle injury (Roth-Johnson 2004; Webster 2005). Hormonal modulation may have been involved in this study, since gonadectomy would have been associated with a reduction in circulating oestrogen. The muscle trauma resulting from abdominal opening during gonadectomy may also have elicited the increased ALT and GGT production. However, the lack of significance between changes in control and treatment group ALT and GGT levels suggests that the hepatic production of these enzymes was not modulated in response to dietary isoflavones, either during exposure or following a 40-day recovery period.

This finding is in contrast to previous findings in cheetahs, in which removal of dietary isoflavones elicited a reduction in ALT (Setchell *et al.* 1987a). However, this earlier study did not control for the variable nutrient composition of isoflavone-containing and isoflavone-free diets. As such, the alteration in ALT cannot be apportioned solely to isoflavones. In the present study nutrient intake was well controlled for, so the failure to demonstrate any significant changes in ALT or differences in GGT production between control and treatment animals further suggests that the reductions in ALT and GGT seen in cheetahs by Setchell *et al.* (1987a) were not isoflavone-specific.

Furthermore, the sensitivity of ALT for detection of hepatic disease is moderate, at best (Jacob *et al.* 2002), and only mild elevations in either ALT or AST were noted in a domestic cat diagnosed with hepatic VOD (Cave *et al.* 2002). The current study utilised a shorter recovery period than Setchell *et al.* (1987a) and gonadectomy occurred at the time that isoflavone exposure ceased, both of which may also explain the disparate results. Likewise, it is possible that the dose used in this study was insufficient to elicit any change in these enzymes, since GGT was only slightly elevated in rats when exposed to genistein doses of 500 mg/kg BW (McClain *et al.* 2006b).

Rats exhibited a mild increase in ALP production after chronic exposure to 500 mg genistein/kg BW (McClain *et al.* 2006b). In this current study, prior to removal of the isoflavones from their diet, the treatment group ALP was higher compared to the control group, although this difference was not statistically significant. Within individuals, the serum ALP concentration was reduced in treatment animals following removal of isoflavones from their diet, whilst control cats exhibited a mean increase in ALP. However, this difference was also not significant, possibly due to the large inter-individual variation and small sample size.

The trend towards increased ALP concentrations following isoflavone exposure, as well as the subsequent reduction in ALP concentration following cessation of dietary isoflavone exposure may indicate that feline production of this enzyme was up-regulated during the period of isoflavone exposure. Indeed, ALP is known to be elevated in cases of cholestasis, or induced by drug or hormone exposure (Roth-Johnson 2004). Cholestasis is unlikely to have occurred in this study, since no changes in GGT concentration (Roth-Johnson 2004) were observed between groups. However, since the endogenous hormone environment could be expected to be comparable between groups, it is feasible that dietary isoflavones were involved in modulation of ALP. Following a 4-week recovery period, rats exhibited significant recovery in the modulation of ALP (McClain *et al.* 2006b). This study also suggests that isoflavones do not exert permanent changes in liver enzyme production in cats.

It must be noted that the sensitivity of ALP to detect hepatic function is relatively poor due to its occurrence in other clinical conditions, short half life, low hepatic stores and presence as several isozymes (Jacob *et al.* 2002; Roth-Johnson 2004; Webster 2005). Therefore the changes detected in association with the presence/absence of dietary isoflavones do not necessarily infer modulation of hepatic function. However, other causes of ALP induction, such as healing fractures, or bone growth (Roth-Johnson 2004), are unlikely to have been aetiological. The rate of bone growth would be expected to be comparable in these age-balanced groups, and no cats suffered bone fractures at any time during the study. In addition, ALP levels in cats are reported to be more specific to hepatobiliary disease since they are less susceptible to drug induction (Webster 2005).

Elevations in ALP were not beyond the normal reference range for cats. However, extreme elevations in hepatic enzymes may be inhibited in cases of chronic liver damage, since reduced hepatic mass results in inefficient enzyme production (Roth-Johnson 2004). This is unlikely to have occurred in this study since ALT would be expected to have been elevated in all individuals as this enzyme is responsive to both mild injury to a large number of hepatocytes, and severe injury to small cell numbers (Roth-Johnson 2004). The lack of difference between groups during the exposure phase, and the observed increase in ALT following cessation of isoflavone exposure indicates that hepatic injury was not associated with isoflavones. Unchanged AST concentration provides further evidence to support a lack of hepatic insult, since elevations of this enzyme, in combination with increased ALT are generally good indicators of hepatic dysfunction in the cat (Roth-Johnson 2004; Webster 2005). Rats exposed to significantly higher doses of genistein failed to exhibit any change in AST concentration (McClain *et al.* 2006b), indicating that changes in this enzyme alone may be an insensitive marker for isoflavone induced hepatic changes or that isoflavones do not affect hepatic enzyme activity.

Bile acid concentration in the serum is used in veterinary medicine to assess hepatic clearance from portal circulation and functional hepatic mass (Roth-Johnson 2004;

Webster 2005). Fasted bile acid concentrations may offer greater specificity for detecting hepato-biliary disease than ALP, ALT or GGT. However, bile acid levels are poorly correlated with histological findings, and may also be elevated in cases of intestinal disease (Roth-Johnson 2004; Webster 2005). Inter-individual differences in gall bladder emptying, gastric emptying rate, intestinal transit rate, ileal bile acid resorption and gut microflora are all known to affect entero-hepatic recirculation of bile acids (Webster 2005). Moreover pre- and post-prandial bile acid concentrations in a domestic cat known to be suffering from hepatic VOD were within the normal reference range for this species (Cave *et al.* 2002). Given the variability of this parameter and the small sample size in this study, it is unsurprising that no difference in bile acid production was detectable between the two groups. In light of the other parameters concurrently evaluated, this finding supports a general lack of effect of dietary isoflavones on hepatic function or enzyme production.

Hepatic adaptation of enzyme production following chronic exposure to isoflavones may have occurred in this study, and between-group differences may be more apparent following acute exposure. However, acute exposure was not measured in the current study. Since cats were exposed to dietary isoflavones at weaning it was not possible to evaluate acute pre- and post-isoflavone exposure responses. Future investigations should include adult cats which have not been exposed to isoflavones, prior to acute dietary isoflavone exposure.

6.2. Experiment Two: Hepatic Histology Following Chronic Ingestion of Genistein and Daidzein

6.2.1. Aim

Hepatic architecture is modulated during veno-occlusive disease (VOD). Histological changes associated with this hepatic disease include hepatic congestion, haemorrhage, hepatocyte and Ito cell vacuolation, foci of extra-medullary haematopoiesis (EMH) (a marker for hypoxia, infection and/or precocious immune response) (Törő *et al.* 2007), and increased neutrophil and macrophage cell numbers. This disease is common in captive cheetahs and has been linked with genistein and daidzein intake (Setchell *et al.* 1987ab). The aim of this study was to determine the effect of chronic oral exposure to the isoflavones, genistein and daidzein on hepatic histology in the domestic cat, as a model for the cheetah.

6.2.2. Materials and Methods

6.2.2.1. *Animals*

Animals are as described in Section 6.1.2.1. Ethical approval for this trial was obtained from the Massey University Animal Ethics Committee (2006).

6.2.2.2. *Liver biopsy collection*

A liver biopsy was obtained from cats that had been exposed to dietary isoflavones for approximately 394 days (see Section 5.1.2.2.) during routine gonadectomy (see Section 5.2.2.2.). Briefly, general anaesthesia was induced with Zoletil 100 (tiletamine and

zolazepam 500 mg/ml each; 12 mg/kg BW, sub-cutaneously) (Virbac, Auckland, New Zealand) and maintained with halothane/oxygen delivered *via* an endotracheal tube. A midline incision was made in order to perform routine ovario-hysterectomy (Section 5.2.2.2). Upon completion of this procedure, a distal liver lobe was located and a wedge biopsy (0.7 – 1 g) taken from its border. One or two catgut sutures were used to control haemorrhage of the liver parenchyma (Cole *et al.* 2002) and routine abdominal closure followed. All animals received temgesic (324 µg/ml buprenorphine hydrochloride, 0.03 mg/kg BW, sub-cutaneously) (Reckitt Benckiser, Auckland, New Zealand) for pain relief after surgery, and ketofen (ketobrofen, 1 mg/kg BW *per os*) for the next 48 h. Cats were maintained in individual metabolism cages for the 14 day period following surgery, after which time sutures were removed and cats were returned to normal housing.

6.2.2.3. *Liver histology*

The liver biopsy was immediately weighed and transferred to 10% neutral-buffered formalin (NBF). Samples were processed on a Leica® TP1050 Tissue Processor (Global Science and Technology, Auckland, NZ). The samples were dehydrated through graded alcohols (70%, 95% and absolute ethanol, BD, Poole, UK) at an ambient temperature, cleared in xylene and impregnated with Paraplast® Tissue embedding Medium (Global Science and Technology, Auckland, NZ) under pressure at 60°C. The samples were then embedded using a Leica Histo Embedder (Global Science and Technology, Auckland, NZ) and 3 µm sections were cut using MicroTec® Rotary Microtome (Global Science and Technology, Auckland, NZ). The sections were floated onto a Thermo® Tissue Bath (Medica Pacifica, Auckland, NZ) at 43°C. The haematoxylin and eosin-stained (H&E) and Masson's Trichrome-stained sections were then mounted onto Superfrost, pre-cleaned slides. The H&E slides were placed in a 60°C oven for 20 minutes then stained on a Leica® Autostainer XL (Global Science and Technology, Auckland, NZ).

The Masson's Trichrome slides were placed in a 60°C oven for 20 min then dewaxed on a Leica® Autostainer XL (Global Science and Technology, Auckland, NZ) and stained.

Following hydration in water, slides were left to mordant in Bouin's solution (Merck, Palmerston North, NZ), overnight at room temperature. Slides were then washed in tap water and stained in Celestine Blue (Merck, Palmerston North, NZ) for 10 min. Slides were rinsed again before staining in Mayer's Haematoxylin (Merck, Palmerston North, NZ) for 10 min and further rinsing. Slides were rinsed for 4 min and then stained in Beibrich Scarlet-Acid Fuchsin (Merck, Palmerston North, NZ) for 2 min before rinsing. Sections were covered in 5% Phosphotungstic Acid (Merck, Palmerston North, NZ) for 15 min and then rinsed prior to staining with Light Green Solution (Merck, Palmerston North, NZ). After rinsing sections were blotted dry with filter paper and placed in 1% Glacial Acetic Acid (BD, Poole, UK). Sections were then blotted dry again before dehydrating in 95% ethanol, absolute ethanol, and finally clearing in xylene before mounting.

Liver sections were examined by a veterinary histo-pathologist. The histo-pathologist was blinded to the treatment groups and provided a detailed report of the sections from each cat. Parameters reported were haemosiderin accumulation, intra-hepatocyte vacuolation, hepatocyte degeneration, necrosis or regeneration. The presence/absence and extent of histological parameters were then tabulated and averaged according to treatment group.

The extent of fibrous tissue development around 3 hepatic blood venules was measured using ImageJ software (version 1.38. Rasband 2007; Research Services Branch, National Institute of Mental Health, MD, USA) and expressed as the percentage of the total area of each blood venule.

6.2.2.4. *Statistical analyses*

Residual data was tested for normality with the Anderson-Darling test and found to be normal. Data was tested for significant differences between fibrous area surrounding

hepatic blood venules in each cat, using a one-way ANOVA. Differences between the incidence of congestion, vacuolation, extra-medullary haematopoiesis (EMH) and inflammatory cells in treatment and control groups were tested with the Fisher's exact test. All statistical analyses were performed with Minitab software (version 15, Minitab Inc., PA, USA). Data are presented as mean (± SEM), unless otherwise stated.

6.2.3. Results

There were few histological abnormalities in any of the liver sections. A lack of haemosiderin accumulation indicated that any observed congestion and periportal haemorrhage was recent. Intra-hepatocyte vacuolation was not significant, and any inter-individual variation in vacuolation was thought to represent divergent glycogen accumulation and reflective of differences in body condition and/or differences in fasting time.

No evidence of hepatocyte degeneration, necrosis or regeneration was observed. One treatment cat had low numbers of neutrophils around some periportal areas, but these were considered unlikely to be significant. Since these were only observed in the cat diagnosed with pyometra (see Section 5.2.3.2.a.), their presence may be related to a systemic inflammatory response.

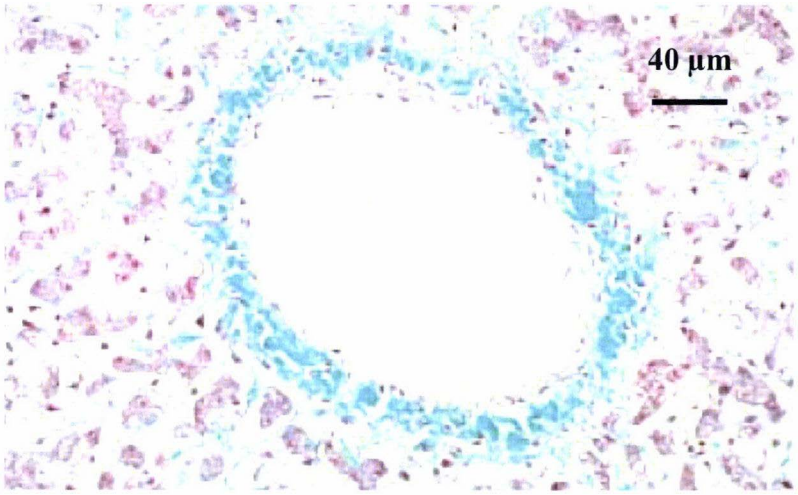


Figure 6.1a. Liver section from a domestic cat with diagnosed hepatic veno-occlusive disease (from Cave *et al.* 2002). Central vein surrounded by subendothelial fibrosis (stained dark green). Masson Trichrome stain.

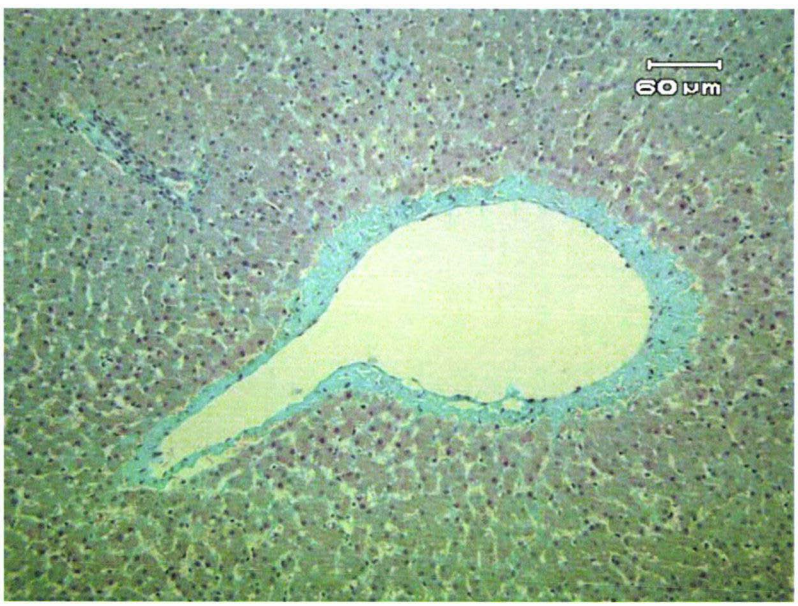


Figure 6.1b. Liver section from a domestic cat in the current study. Central vein surrounded by subendothelial fibrosis (stained green). Massons Trichrome stain.

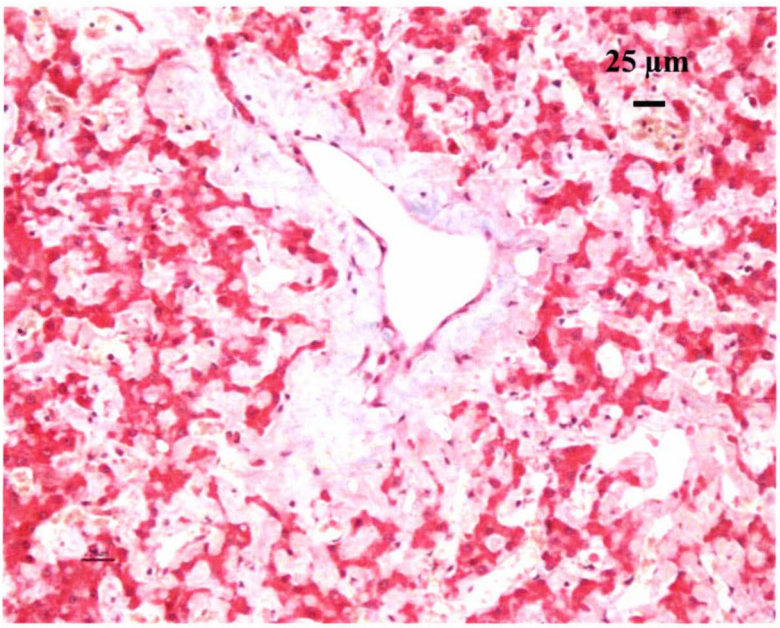


Figure 6.1c. L liver section from a cheetah with veno-occlusive disease (Courtesy Wellington Zoo). Central vein surrounded by subendothelial fibrosis (stained blue). Massons Trichrome stain.

Figures 6.1a and 6.1b show sections of cat liver tissue containing hepatic venules. The blue staining surrounding the venule represents the extent of fibrous collagen tissue. The mean area of fibrous tissue surrounding hepatic blood venules in control cats represented 28.51% (± 2.60%; range 14.78 – 40.05) of the venule area, and that of treatment cats was not significantly different (32.84 ± 4.18%; range 20.72 – 51.16, $p > 0.05$). A liver section from a domestic cat suffering from hepatic VOD was also measured from a published photomicrograph and found to exhibit fibrosis covering an area equivalent to 46.75% of the venule lumen area (Figure 6.1a). Over half (57%) of the control cat sections, but all (100%) of treatment cat sections demonstrated congestion, however this difference was not significant ($p > 0.05$). No significant difference in the incidence of vacuolation of hepatocytes and Ito cells could be found between control cat sections (86%) or treatment cat sections (50%) ($p > 0.05$). Extra-medullary haematopoiesis was detected in 14% of control cat sections and 25% of treatment cat sections, but no difference was detectable ($p > 0.05$). Neutrophils and/or macrophages were not observed in any control cat section, but were seen in 50% of treatment cat sections. However, this difference also failed to achieve statistical significance ($p > 0.05$).

A summary of the histological findings is provided in Table 6.3.

	Control Group	Treatment Group
Congestion	57%	100%
Vacuolation	86%	50%
Extra-medullary haematopoiesis	14%	25%
Neutrophils/macrophage infiltration	0%	50%
Fibrous area	28.9%	32.8%

Table 6.3. Summary of mean hepatic parameter scores in the liver biopsies of control (N = 7) and treatment cats (N = 4) at the time of ovario-hysterectomy. Data is presented as the percentage of cats with positive observation scores for each parameter. The exception to this is fibrous area which is presented as the mean area of fibrous tissue surrounding hepatic blood venules as a percentage of the venule area.

6.2.4. Discussion

Sinusoidal and haemorrhagic congestion with perivenular fibrosis are reported to be typical histological signs of veno-occlusive disease (VOD) in cheetahs (see Figure 6.1c) and the domestic cat (see Figure 6.1b) (Setchell *et al.* 1987a; Cave *et al.* 2002). Veno-occlusive disease in cheetahs has been postulated to be associated with dietary isoflavone exposure (Setchell *et al.* 1987a). However, no evidence could be found for isoflavone-induced hepatic congestion, vacuolation, EMH or inflammatory cell infiltration in domestic cats following extended exposure to dietary isoflavones. Although a greater proportion of cats in the treatment group, compared to the control group, demonstrated hepatic congestion (100% *versus* 57%, respectively), this failed to achieve statistical significance, and was most likely due to anaesthesia and surgical procedures, rather than any underlying hepatic disease (W. Roe, pers. comm.).

Similarly, no evidence of hepatic fibrosis or pathology was detected in any cat, regardless of treatment group. The extent of hepatic fibrous tissue formation around blood venules did not differ between groups, which suggests that if dietary isoflavones act in a similar

manner between cheetahs and cats, these compounds are not likely to play an aetiological role in the VOD observed in captive cheetahs, as was hypothesised by Setchell and co-workers (Setchell *et al.* 1987ab; Gosselin *et al.* 1988).

Hepatic VOD has only been reported in one domestic cat, but the aetiology was undetermined (Cave *et al.* 2002). The liver of this cat was reported to exhibit increased masses of subendothelial collagen fibres around the central veins and in the tunica media of larger sublobular veins. One cat in the treatment group of this study exhibited a greater degree of fibrosis around one vein than was measured from the photomicrograph reported by Cave *et al.* (2002). This measurement appears to be an outlier as no other venules from any cat included in this trial exhibited the extensive fibrosis seen in the individual reported by Cave *et al.* (2002). However, further investigation with a larger sample size may be warranted.

Budd-Chiari-like syndrome is the rare condition typified by hepatic venous outflow obstruction (Cave *et al.* 2002), potentially related to VOD. The condition has only been reported in two domestic cats, but 24 dogs are known to have suffered from it (Cave *et al.* 2002). Elevations in tumour necrosis factor-alpha (TNF-α) and Interleukin-1β are observed in association with this syndrome, although it is unclear as to whether they play an aetiological or responsive role (Cave *et al.* 2002). Interestingly, the ability of isoflavones to inhibit TNF-α (Kang *et al.* 2005), suggests that dietary isoflavone intake is more likely to reduce, rather than increase, the risk of hepatic fibrosis and VOD in domestic cats. Such a protective mechanism has also been postulated in other studies (Kang *et al.* 2001; Liu *et al.* 2002). Although this study was not designed to assess cellular proliferation or hepatic toxicity, the lack of difference between control and treatment animals indicate that neither beneficial nor detrimental effects were elicited in the liver following isoflavone exposure, under the conditions of this trial.

Hepatic fibrosis is a dynamic process, involving nonspecific mechanisms which respond to inflammation and/or hepatic injury (Center 2004). Changes in liver architecture are primarily due to the deposition of extracellular matrix which operates to reduce perfusion and stimulate sinusoid capillarisation and collagenisation (Center 2004). The chronic nature of the appearance of these effects indicates that differences between treatment and control animals may not have become detectable until much later in life. Therefore, the use of larger sample sizes and/or exposing cats to isoflavones for a longer period of time (perhaps a lifetime) may be necessary to determine the potential for isoflavone modulation of hepatic fibrosis incidence. Additionally, the validity of the domestic cat as a model for the cheetah in this instance may be doubtful due to the rarity of VOD in domestic cats compared to its high prevalence in captive cheetah populations.

6.3. Conclusions

- No significant differences were detectable in hepatic biochemistry between treatment and control groups, and all serum values were within the normal reference ranges for domestic cats.

- However, a trend towards divergent ALP levels was observed, and future studies with larger sample sizes may provide clearer indication of a role for dietary isoflavones in modulating hepatic biochemistry.

- The fibrosis observed in one treatment animal, which was more severe than seen in a domestic cat known to be suffering from hepatic VOD, may be an incidental finding. However, treatment animals demonstrated slightly (but not significantly) greater areas of fibrosis surrounding hepatic venules than control animals. The potential exists that longer term exposure to isoflavones may be associated with hepatic fibrosis in domestic cats.

- Differences between groups in hepatic venule fibrosis may have reached statistical significance if a larger sample size had been used. The extension of this trial into mid- or late-life stages may, likewise, have demonstrated divergent results.

- On the basis of the results presented, dietary isoflavones, at the current dose and duration of exposure do not appear to modulate hepatic enzyme production or histological parameters. Nonetheless, further investigation of lifetime exposure in a larger population of cats is warranted.

CHAPTER SEVEN:

Overall Discussion

Initial experiments during this project resulted in the identification and quantification of four dietary isoflavones in feline diets available in New Zealand. These results agreed with previously published reports for companion animal diets (Court and Freeman 2002; Cerundolo *et al.* 2004). However, a major difference from these earlier experiments was the observation that diets which did not list soy as an ingredient were occasionally found to contain isoflavones. Court and Freeman (2002) reported that the presence of soy in commercially prepared diets was positively correlated with both price and isoflavone content. Yet, the findings of this thesis indicated that diets available in New Zealand may either contain isoflavones derived from non-soy ingredients, or that differences in statutory labelling requirements meant that soy was utilised but not declared by New Zealand manufacturers.

Setchell *et al.* (1987a) indicated that the majority of commercially prepared North American diets consumed by cheetahs in captivity contained soy protein and/or isoflavones. The survey of zoo diets included in this thesis supported this suggestion, and found that over 60% of the zoo-specific diets contained some form of isoflavone. However, only one of the six diet formulations was found to contain anywhere near the concentrations of isoflavones thought to be capable of eliciting physiological changes in mammals. Furthermore, the diet thought to be the most widely used for feeding captive cheetahs in North America (Dierenfeld 1993; Bechert *et al.* 2002), contained negligible concentrations of genistein and daidzein. Hence, captive cheetah exposure to dietary isoflavones cannot be generalised and, in fact, may only be of concern in facilities utilising one specific diet brand. Therefore, the suggestion by Setchell *et al.* (1987a) that isoflavones may be aetiological in liver and reproductive disease in the general captive cheetah population appears unlikely. On the other hand, what was of particular concern was the finding that a popular milk replacer for neonatal cheetahs contained isoflavones in concentrations which would expose cubs to intakes up to 4 times greater than consumed by adult cheetahs fed on any diet tested. This level of intake has been associated with significant perturbations in growth and development, often with long term health consequences in other species.

Although the experiments conducted during this thesis did not include investigation of the bioavailability of isoflavones in felids, the fraction of genistein detected in the plasma of domestic cats here was greater than the bioavailability reported by Cave *et al.* (2007a) who measured true bioavailability. It is possible that this may be related to the higher dose used by Cave *et al.* (2007a) and would support the existence of a saturation threshold in absorptive mechanisms. Studies reported here indicate an enhanced absorption of both genistein and daidzein in cats, compared to other species.

Experimental results determined the previously unknown ability of the domestic cat and cheetah to metabolise genistein and daidzein by conjugation to a sulphate moiety. Moreover, the production of the potentially more potent daidzein metabolite, equol, was also reported in these species for the first time. As supported by the literature, cats and cheetahs did not produce detectable concentrations of glucuronide metabolites (Hartiala 1955; Robinson and Williams 1958; Trepanier *et al.* 1998; Court and Greenblatt 2000), but instead utilised sulphate conjugating pathways to metabolise these phenolic compounds. This finding draws doubt over the suggestions made previously regarding the potentially increased susceptibility of felids to dietary isoflavone action due to their poor glucuronidation capacity (Setchell *et al.* 1987a; Court and Freeman 2002). It now appears that the metabolising capacity of the domestic cat and cheetah is within the range reported in other species tested, although the cheetah may be slightly inferior to the cat in this regard. The greater proportion of unbound metabolites detected in the plasma of cheetahs may indicate that the strength, direction or frequency of isoflavone-induced biological changes may be increased in this endangered species, compared to its household counterpart. Yet, having said that, isoflavone absorption in the cheetah appears to be impaired compared to the domestic cat, which may counteract any interspecific difference in overall effect.

Cheetah cubs, tested at what is assumed to be steady state, excreted a greater proportion of unbound isoflavones in their urine than were detected in the urine of adults after a

single bolus. This may have been due to a higher dietary exposure level or variable duration of exposure, although other authors have suggested a reduced metabolic capacity toward isoflavones in neonatal humans (Setchell *et al.* 1997) so further testing is necessary. Since isoflavones may exhibit enhanced biological effects in neonates, potentially due to reduced conjugation capacity or the critical stages of development occurring during this lifestage, *in utero* and/or neonatal exposure may confer divergent effects and warrants investigation. Therefore, controlled mating, conception, gestation and offspring-rearing studies are required to determine the long-term effects of isoflavones on feline reproductive capacity and health.

Additionally, these results have provided an indication that the metabolites produced by felids may vary according to dietary form, and between acute and chronic ingestion in the cat. It is possible that differences in conjugation capacity between the different forms of isoflavones administered to domestic cats may be dose-related, and could reflect the presence of a saturation threshold in the phase II metabolic pathways. This observation requires further investigation as determination of a dose-response was not an objective of this thesis.

Although the cheetah and domestic cat did not show identical plasma metabolite profiles or excretory patterns following ingestion of equivalent doses, they did demonstrate a commonality in equol production, sulphate conjugation and lack of glucuronide production. Moreover, the extent of conjugation observed in the cheetah appears to be better reflected in the domestic cat than it would be if comparisons were made with any other mammalian species for which data exists. Consequentially, it appears that the domestic cat provides the most suitable model for the prediction of isoflavone-related physiological changes in captive cheetahs consuming isoflavones.

Chronic ingestion of dietary isoflavones (at doses achievable in a normal felid diet) did not influence oestrous cycle characteristics, uterine or ovarian wet weight in female

domestic cats. Likewise, vaginal cytology was unaffected by dietary isoflavones, which was consistent with previous findings where a dose of 100 mg/kg BW was necessary to demonstrate oestrogen-like changes in vaginal cytology in the cat (Cave *et al.* 2007c). Nonetheless, as has been reported for other species, dietary isoflavones were successful in modulating histological and molecular parameters in cats, in the absence of gross uterotrophic effects (Badger *et al.* 2001; Nikaido *et al.* 2005). Furthermore, the increased luminal epithelial cell height observed in isoflavone-treated cats indicated a hypertrophic effect, in spite of a lack of effect on overall uterine wet weight. Hence, although uterotrophic studies, most frequently including only organ wet weight measurements, are typically considered to be the "gold-standard" for detection of oestrogenic effects by isoflavones, this parameter was found to be an insensitive and unreliable marker for isoflavone-related biological activity in the cat. The tissue- and receptor-specific differences in response to isoflavone exposure observed in the studies of this thesis have also been reported in the literature (Calderelli *et al.* 2005; Hertrampf *et al.* 2005; Brožič *et al.* 2006; Diel *et al.* 2006; Limer *et al.* 2006) and highlight the importance of monitoring multiple endpoints.

As an indirect consequence of this study, the previously unreported distribution of ERβ was determined. Interestingly, histological changes were more apparent in response to isoflavone exposure in the endometrium, suggesting a potential role for ERα-mediated pathways in isoflavone activity due to the higher ERα expression in this tissue. Isoflavones may act in an oestrogen-like manner when ERα receptor content is high, but opposing effects to those observed for oestrogen may be more likely to occur when ERα content is reduced. This may indicate that the anti-oestrogenic effects are mediated *via* ERβ-mediated pathways, for which isoflavones have a greater binding affinity (An *et al.* 2001; Bennetau-Pelissero *et al.* 2001; Ford *et al.* 2006; Chrzan and Bradford 2007).

Isoflavones are known to exert influence over the demonstration of sexually-related behaviours in rodents (Patisaul *et al.* 2001; Kouki *et al.* 2003; Kudwa *et al.* 2007), and a trend towards the increased demonstration of sexual behaviour was also observed in cats

following chronic ingestion of dietary isoflavones. Although this requires further testing, it is possible that isoflavones may have been acting within the neuroendocrine system of the cat. It is pertinent to note again that reliance on uterotrophic effects to demonstrate oestrogenicity may be misleading. Setchell *et al.* (1987a) mentioned acyclicity as a potential outcome of isoflavone exposure in captive cheetahs and interference at the hypothalamic level in felids now appears worthy of further investigation. Additionally, the potential for increased spontaneous ovulation events (observed here in domestic cats) would provide a possible explanation for the reports of irregular or interrupted cyclicity in the captive cheetah population. This requires more in-depth investigation as sample sizes were limited in these studies. Similarly, the determination of the stage of oestrous, spontaneous ovulation and sexual behaviour need better validation in future studies.

In regards to the effect of isoflavones on hepatic health, changes were less obvious than observed in the reproductive tract. Dietary isoflavones, at the described dose and duration of exposure, did not appear to modulate hepatic enzyme production or histological parameters. This is in contrast to the findings of previous studies (Setchell *et al.* 1987a; Gosselin *et al.* 1988), but issues regarding the trial design used in these previous studies may have contributed to this discrepancy. Nonetheless, further investigation of lifetime isoflavone exposure in a larger population of domestic cats and cheetahs is warranted.

Overall, observations reported in this thesis for cats suggested that isoflavones consumed by captive cheetahs via commercially prepared diets are unlikely to contribute significantly to veno-occlusive disease. However, the apparently greater availability of unbound forms of genistein and daidzein in the cheetah, compared to the cat, indicates that biological activity may be enhanced in the cheetah. As a result, the modulated sex steroid receptor expression and increased luminal epithelial cell height observed in domestic cats may also occur in cheetahs; potentially with greater magnitude. The on-going investigation of potential factors involved in reproductive and hepatic dysfunction

or disease in the captive cheetah is critical to the establishment of a self-sustaining captive population of this species.

Although the findings of this thesis require further investigation, support has been demonstrated for the possible association between dietary isoflavones and changes in the reproductive system of felids. Given the endangered status of the cheetah and the potential mechanisms of activity indicated here, it would be prudent to include dietary isoflavones as a test variable in future studies of cheetah reproduction and fertility. In the interim, it would be advisable to remove all isoflavone-containing milk replacer products from use within zoos for the hand-rearing of neonatal cheetahs. Additionally, zoos using zoo-specific diet #2 are encouraged to consider substituting its use for an alternative non-isoflavone containing diet until further research can be conducted. The use of high isoflavone-containing diets for domestic cats is also contra-indicated until further research is conducted to determine its safety.

Future work should be directed at establishing the existence of any epidemiological links between dietary isoflavone intake and impaired reproductive performance or disease prevalence in captive cheetahs (including post-mortem data). The results of this epidemiological work should then be used to design non-invasive intervention trials in captive cheetahs. Further work is warranted in determining the effect of chronic isoflavone intake on domestic cat fertility and fecundity and controlled mating and kitten rearing studies are suggested starting points for this. The validity of the domestic cat as a model for hepatic health in cheetahs deserves investigation.

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APPENDIX ONE:

Publication of Results from Section 2.1

APPENDIX TWO:

Erratum to Publication of Results from Section 2.1

Corrigendum

Re: The isoflavone content of commercially-available feline diets in New Zealand

The paper by KM Bell, SM Rutherfurd and WH Hendriks published in the *New Zealand Veterinary Journal* 54, 103–108, 2006, entitled, "The isoflavone content of commercially-available feline diets in New Zealand" contained an error in the content of Table 2 in which the values for the "Moist with-soy" and "Dry without-soy" categories of diet were transposed. The correct table is:

Table 2. Total content of isoflavone of feline diets and predicted intake of isoflavone, according to category of diet[a]. Medians and percentiles reported as data were not normally distributed.

	Total isoflavone content (mg/kcal ME)[b]				Predicted isoflavone intake of domestic cats (mg/kg BW)[c]			
Category of diet[a]	Minimum	Median	Upper quartile (75%)	Maximum	Minimum	Median	Upper quartile (75%)	Maximum
Moist meat-only	0	0.0004	0.0007	0.0022	0	0.03	0.06	0.17
Moist without-soy	0	0.0007	0.0015	0.0685	0	0.05	0.12	5.48
Moist with-soy	0.0130	0.0184	0.0246	0.0284	1.04	1.47	1.96	2.27
Dry without-soy	0	0.0022	0.0297	0.1017	0	0.18	2.38	8.13
Dry with-soy	0	0.0036	0.0396	0.0583	0	0.29	3.17	4.66

[a] Diets were categorised according to their dry matter (DM) content (dry >90% DM; moist <25% DM), the presence/absence of non-soy plant material, and the presence/absence of soy, according to the manufacturer's labelling. Since all diets were found to contain some form of non-soy plant material, except for the diets categorised as 'moist meat-only', this exclusion criterion was not included in the name of each category of diet.

[b] The metabolisable energy (ME) content of each diet analysed was estimated using the modified Atwater factors (crude protein x 3.5, crude fat x 8.5, and carbohydrate x 3.5). The contents of protein, fat and carbohydrate were recorded as per the manufacturer's labelling, and where the content of carbohydrate was not reported this was estimated by subtraction (Anonymous 2004).

[c] The intake of isoflavone of domestic cats was estimated according to the total content of isoflavone of each diet per unit of ME. According to the National Research Council (Anonymous 1986), active domestic cats have an approximate ME requirement of 80 kcal/kg bodyweight (BW). Under this assumption, the total content of isoflavone occurring in 80 kcal of each diet is equivalent to the total content of isoflavone consumed by a domestic cat per kg BW

APPENDIX THREE:

Publication of Pilot Study to Section 3.1, 3.2 and 3.3

APPENDIX FOUR:

Publication of Results from Section 5.1

Concurrent Session 12: Animal Nutrition

Genistein and daidzein do not affect puberty onset or oestrus cycle parameters in the domestic cat (*Felis catus*)

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Background – Dietary isoflavones (genistein and daidzein) possess oestrogenic properties and are present in commercially available feline diets. These isoflavones are reported to influence the reproductive system in a variety of mammalian species, although evidence is disparate and conflicting.

Objective – To determine if dietary isoflavones influence the onset of puberty and oestrus cycle parameters in the domestic cat when consumed during the developmental period.

Design – Kittens were maintained on either treatment (base diet + 300μg/g DM isoflavones, n=6) or control (base diet + vehicle, n=9) diets for up to 480 days post-weaning. Vaginal smears were taken thrice weekly and examined for oestrogen-induced cellular degradation. The first sign of oestrogen activity, onset of regular cycling, duration of oestrus and inter-oestrus periods and the incidence of spontaneous ovulation (inter-oestrus periods >20 days) were recorded and compared between groups.

Outcomes – No significant difference ($P>0.05$) was found in the onset of puberty or any oestrus cycle parameter examined. However, cats in the treatment group demonstrated a significantly greater incidence of spontaneous ovulation compared to control group cats (13.6% versus 3.9% of observed inter-oestrus periods, respectively; $P=0.03$).

Conclusions – Genistein and daidzein, when consumed at levels representative of commercially available feline diets, do not alter puberty onset or oestrus cycle parameters in the domestic cat. However, the greater incidence of spontaneous ovulation induced by these isoflavones may be of clinical significance and warrants further investigation.

APPENDIX FIVE:

Publication of Results from Section 5.2

be due to intrauterine inseminations at two different points. In the future studies, it is suggested that, non-surgical intrauterine insemination techniques are needed to be tried in queens.

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Assessment of reproductive histology and sex steroid receptor expression in the domestic cat (Felis catus) following chronic exposure to phytoestrogens

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Phytoestrogens are secondary plant compounds, incorporated into commercially prepared felid diets through the use of soy-derived ingredients. The phytoestrogens genistein and daidzein, have been shown to elicit changes in reproductive tract histology and sex steroid receptor expression in a variety of mammalian species. These phytoestrogens are considered to be potential aetiological agents in the infertility suffered by a large percentage of the captive cheetah (Acinonyx jubatus) population. To investigate this hypothetical role in felid reproduction, domestic cats (n = 6, 4 female, 2 male) were exposed to dietary genistein and daidzein (300mg/g DM total isoflavones) from weaning (8 weeks of age) until approximately 15 months of age. A control group of related cats (n = 10, 8 female, 2 male) were reared under identical conditions and fed the same diet without the addition of phytoestrogens. Reproductive tracts were collected from all animals during routine gonadectomy and processed for histological and immunohistochemical analysis (IHC). Tissues were collected from female animals (mean age 485 days) during inter-oestrous as assessed by vaginal cytology, and from male animals at 350 days of age. Reproductive tracts were assessed for histopathological changes, and other parameters including uterine luminal epithelial cell height and follicular development in females. The expression and distribution of Estrogen Receptor (ER)-α, ERβ and Progesterone Receptor (PR) was assessed in ovarian and uterine tissue using IHC techniques. Wet weight of the reproductive tracts did not differ between groups and all but one tract (treatment group) were within normal expected range of inflammatory cell infiltration. Luminal epithelial cell height was greater in treatment animals (5.39μm vs. 4.36μm, $p < 0.05$) but no differences were detectable in ovarian histology. Expression of ERα and ERβ was up-regulated in the tracts of treatment animals, whilst PR expression was generally down-regulated, compared to controls ($p < 0.05$). Tissue- and receptor-specific variation was apparent. Although isoflavones were not found to be uterotrophic, the observed histological changes were suggestive of oestrogenic activity. Furthermore, the ability of isoflavones to modulate the proportional expression of sex steroid receptors may have implications for fertility and fecundity in later life. Future investigations in domestic cats utilising larger sample sizes, regimes including in utero and/or lactational exposure and controlled fertility and fecundity testing are warranted.

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The effect of GnRH on fertility of alpacas inseminated with frozen-thawed semen

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Gonadotropin releasing factor (GnRH) was used immediately after artificial insemination with frozen-thawed semen in alpacas. GnRH diluted in Tris buffer was deposited in the uterine horn ipsilateral to the ovary containing an ovulatory-sized follicle. Ovulation was induced with hCG (Chorulon, Intervet) 24 hours before artificial insemination in 157 adult female alpacas that were divided into three groups: 58 control, 43 with 0.1 μg GnRH, and 56 with 1 μg of GnRH (Fertagyl, Intervet). There was no difference in ovulation percentage between the three groups, with 72.6% of females ovulating. There was no significant difference in pregnancy at 21 days between the two dosages of GnRH, with 75%, and 76.2% of females being pregnant for 0.1 μg and 1.0 μg GnRH, respectively; however, 65% of females of the control group were pronounced pregnant, which is significantly different than the females that received GnRH ($P < 0.05$). The ovulation percentage was in agreement with previous reports for alpacas. This study suggests a paracrine effect of GnRH, when used at the time of insemination of female alpacas with frozen-thawed semen, that might be used in programs of artificial insemination in this species.

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Distribution of oestrogen receptor alpha and progesterone receptor and leukocyte infiltration in canine cervical tissue

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Introduction Cervix serves as a physical barrier of female reproductive tract to prevent ascending infection by mucus secretion and constriction. Changes of the cervical patency are cycle-dependent. In woman, cervical dilatation at labouring is correlated with the extent of neutrophils infiltration. However, mechanisms of cervical dilatation in the bitches are not well understood. This study aimed to investigate the distribution of oestrogen receptor α (ERα) and progesterone receptor (PR) and leukocyte infiltration in cervical tissue of normal bitches during various stages of the oestrus cycle and bitches undergone open- and closed-cervix pyometra.

Methods Cervical tissues were collected from bitches subjected to ovariohysterectomy and divided into six groups: inactive (19); follicular (16); early luteal (7); luteal (13); open-cervix pyometra (22) and closed-cervix pyometra (19). The samples were fixed in 4% paraformaldehyde, embedded in paraffin blocks, longitudinally sectioned and placed on two slides. One slide was stained with haematoxyline-eosin and evaluated for leukocyte infiltration. The other was processed for immunohistochemical evaluation of ERα and PR. The cervical tissue was divided into two parts: the uterine part was characterised by a simple columnar epithelium and the vaginal part was characterised by a stratified squamous epithelium. Three tissue layers were evaluated: the surface epithelium; the propria submucosa and the tunica muscularis. An immunohistochemical total score consisted of the addition of intensity and proportional score. Leukocytes were quantified in five microscopic fields of 0.0845 mm^2. The statistical models included the effects of groups, tissue layers and cervical parts. The immunohistochemical scores were compared using Kruskall-Wallis test. Differences of the number of leukocytes were analysed and compared using ANOVA and Tukey test.

Results The ERα and PR scores were different between groups and between layers ($P < 0.05$) but not between parts ($P > 0.05$). Lower ERα and PR scores ($P < 0.05$) and higher numbers of leukocytes ($P < 0.05$) were observed in the pyometra groups than the normal bitches. Differences of the ERα and PR scores were not seen between the open- and closed-cervix pyometra ($P > 0.05$) whereas higher number of neutrophils was found in the open-cervix than closed-cervix pyometra ($P < 0.05$).

Conclusions The ERα and PR expressions in the cervix of dogs are influenced by stages of the oestrus cycle. Neutrophils infiltration in the cervical tissue appears to involve in cervical dilatation in pyometra bitches.