

# 1 **PILOT STUDY**

2

## 3 **Preliminary investigation of the influence of long-term dietary isoflavone intake on** 4 **reproductive tract histology and sex steroid receptor expression in female domestic** 5 **cats (*Felis catus*)**

### 6 **Summary**

7 Genistein and daidzein are isoflavones which are reported to influence the reproductive  
8 system in a variety of mammalian species. This pilot study aimed to determine if dietary  
9 isoflavones could potentially influence reproductive tract histology or morphology in  
10 domestic cats, when consumed during the postnatal development period. Cats were  
11 maintained on either treatment (150 µg/g DM genistein and 150 µg/g DM daidzein, n=4)  
12 or control (isoflavone free, n=8) diets from weaning, up to 414 (± 17.2) days post-  
13 weaning. Reproductive tissues were collected during routine ovario-hysterectomy and  
14 examined for histology and sex steroid receptor expression. Findings indicate that these  
15 dietary isoflavones influenced the expression of oestrogen receptor  $\alpha$  (ER $\alpha$ ) and  
16 oestrogen receptor  $\beta$  (ER $\beta$ ), and progesterone receptor in feline reproductive tissues. One  
17 cat in the treatment group developed suppurative endometritis, but no evidence of  
18 uterotrophic or histological changes were found in any other cats. The potential to alter  
19 expression of hormone receptors in the reproductive tract of domestic cats exposed to  
20 genistein and daidzein warrants further investigation.

21 **Keywords:** cat, daidzein, genistein, oestrogen, progesterone, reproduction

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## 24 **Introduction**

25 Dietary isoflavones are phenolic compounds found in soy and other legumes, which have  
26 oestrogenic and anti-oestrogenic properties (Kurzer and Xu, 1997). A diverse array of  
27 hormonal and non-hormonal effects have been observed in isoflavone-exposed rodents,  
28 pigs and humans, including oestrogen receptor binding, and morphological and  
29 histological changes (Barnes *et al* 2000; Ford *et al* 2006; McClain *et al* 2006).

30 Domestic cats ingest, absorb, and metabolise soy isoflavones present in commercial diets  
31 (Bell *et al.*, 2006; Cave *et al.*, 2007). Some feline diets expose cats to isoflavone  
32 concentrations potentially capable of eliciting physiological changes (i.e. > 2 mg/kg BW)  
33 (Bell *et al* 2006). The isoflavones, genistein and daidzein, comprise the isoflavones  
34 detected in the highest concentrations in commercially prepared cat food (Bell *et al*  
35 2006). Thus, it is important to ascertain the reproductive consequences of this level of  
36 genistein and daidzein exposure in this species. The present study was conducted to  
37 determine the potential for genistein and daidzein to alter reproductive parameters in the  
38 domestic cat, when provided at concentrations reflective of normal dietary exposure.

## 39 **Materials and Methods**

40 Eighteen female short-haired, domestic cats (*Felis catus*) were enrolled in the study and  
41 assigned to either the control group (n = 9) or treatment group (n = 9) at weaning.  
42 However, six cats were removed from the trial (two due to failure to consume the test diet  
43 within the first week, and four due to unrelated medical conditions prior to planned tissue  
44 collection). Cats were group-housed in multi-level pens, exposed to natural day/night

45 cycles. At 10 weeks of age, the cats were removed from the queen's pens, and separated  
46 into treatment (mean age  $72 \pm 1.89$  d; BW  $0.87 \pm 0.07$  kg) and control (mean age  $71 \pm$   
47  $2.39$ d; BW  $0.93 \pm 0.05$  kg) groups. Ethical approval was obtained from the Massey  
48 University Animal Ethics Committee.

49 The basal diet for both groups was a moist feline diet, commercially-prepared and  
50 formulated to meet the requirements for growth in the domestic cat (AAFCO 2009). This  
51 diet was assayed to contain no detectable levels of isoflavones (Bell *et al.*, 2006). The  
52 purified (99.9%) form of each isoflavone, genistein and daidzein (LC Laboratories, MA,  
53 USA), was added to the basal diet to provide a calculated dose of  $300 \mu\text{g}$  total  
54 isoflavone/g DM. Samples of the control and treatment diets were assayed for isoflavone  
55 content at monthly intervals throughout the trial according to methodology described in  
56 Bell *et al.* (2006). Cats were provided water *ad libitum* during the trial, and offered  
57 enough food to provide each cat with appropriate energy intake for age (i.e.  $217 \text{ kcal/kg}$   
58  $\text{BW/d}$  at eight weeks old, gradually reducing to  $88 \text{ kcal/kg BW/d}$  by 40 weeks; Legrand-  
59 Defretin and Munday, 1993). Food was weighed before and after offering to each group  
60 and daily refusals were used to calculate intake per pen, which was then used to estimate  
61 intake per cat. Monthly assessments were made of individual food intake by separation of  
62 each cat into individual metabolism cages for a 24 hour period, during which time food  
63 was offered in quantities calculated to provide twice the cat's energy needs, and food  
64 intake and urinary and faecal output were recorded.

65 Vaginal cytology confirmed that cats were in inter-oestrous at the time of reproductive  
66 tract collection. Tissue collection was performed under halothane anaesthesia, according  
67 to standard veterinary procedures, at a mean age of 481 days (SEM 21.4) in the control

68 group, and 429 (SEM 62.9) in the treatment group (differences due to older cats in the  
69 treatment group being removed from the study). Surgical procedures were performed on  
70 all cats in both groups at the same point in time (across two days) and by the same  
71 surgeon. Reproductive tracts (including ovaries, uterine horns and cervix) were weighed  
72 and ovarian surfaces examined for the presence of visible follicles, corpora lutea, and  
73 corpora haemorrhagica. Reproductive organs were fixed in 10% buffered formalin  
74 before being processed for histology and immunohistochemical (IHC) analysis.  
75 Haematoxylin and eosin-stained slides were examined by a veterinary histopathologist  
76 (author; W.R.; blinded to treatment) and screened for abnormalities according to standard  
77 veterinary procedures.

78 An IHC assay for assessment of ER $\alpha$ , ER $\beta$  and progesterone receptor (PR) was  
79 developed from the method of Martin de las Mulas *et al* (2000). The ER $\alpha$ , ER $\beta$  and PR  
80 were identified using monoclonal mouse antibodies, IgG1 (NCL-ER-6F11, NCL-ER $\beta$ ;  
81 Vision Biosystems, Victoria, Australia) and PR4-12 (Merck, Palmerston North, New  
82 Zealand), respectively) validated for use in feline tissue.

83 Histological sections were de-paraffinised and rehydrated by sequential immersion in  
84 xylene and graded alcohol baths. Sections were microwave-heated (high power, 750W  
85 microwave oven for 7 min) in citrate buffer (10 mM citric acid, pH 6.0). Sections were  
86 washed in phosphate-buffer solution (PBS) before non-specific binding sites in the tissue  
87 were blocked by the application of 100  $\mu$ l of 10% Bovine Serum Albumin (BSA) (Roche  
88 Diagnostics, Mannheim, Germany) in ovine serum and PBS. Sections were incubated at  
89 room temperature in a moist chamber for 45 min before BSA/serum was removed and the  
90 primary antibody applied (ER $\alpha$ , ER $\beta$  or PR) at dilutions confirmed in preliminary

91 experiments to yield optimal results (1: 50 for ER $\alpha$  and ER $\beta$ , 1: 30 for PR). Sections  
92 were incubated overnight at room temperature before the primary antibody was removed  
93 and the tissues incubated with biotinylated goat, anti-mouse IgG (Invitrogen Life  
94 Technologies, Auckland, NZ) for 1 hour before a fluorescent marker was added  
95 (Streptavidin, Alexa Fluro 546 conjugate, Molecular Probes Inc., OR, USA, diluted 1:  
96 20). Following a final incubation of 1 hour, slides were washed in cold tap water and  
97 counter-stained with haematoxylin.

98 Positive control tissues (control cat uterine tissue and human breast cancer tissue) were  
99 incubated with each of the three primary antibodies and processed according to the same  
100 methodology as test tissues. Tissues were examined by one investigator (author; KW-T)  
101 using 40 x magnification with epi-fluorescence illumination (488 nm), and 100 individual  
102 cells were analysed for fluorescence-staining intensity and extent using Java-based image  
103 processing software (ImageJ, version 1.38; Rasband 2007). The level of light staining  
104 intensity detected in negative control tissue was the threshold of background  
105 luminescence used to define positive staining in test sections.

106 For the statistical analysis, data that were not normally distributed were tested for  
107 differences between groups using the Mann-Whitney test. For proportional data the  
108 Fisher exact test was used to compare differences. All other parameters were tested for  
109 between-group differences using ANOVA. All statistical procedures were carried out  
110 with Minitab software (version 15, Minitab Inc., PA, USA) with confidence limits set at  
111 95%.

## 112 **Results and Discussion**

113 Four cats consumed the treatment diet and eight cats consumed the control diet for the  
114 duration of the study. By the end of the trial, treatment cats were consuming an average  
115 of 4.88 – 5.19 mg total isoflavones/kg BW/d, providing approximately equal doses of  
116 2.44 – 2.56 mg/kg BW/d of genistein and daidzein.

117 No significant differences were observed between groups in reproductive tract wet weight  
118 (Table 1), indicating no gross morphological changes following isoflavone exposure.

119 **Table 1 here**

120 This is in contrast with previous studies in other species in which uterine hypertrophy has  
121 been reported following exposure to isoflavones in rats (Santell *et al.* 1997; McClain *et al.*  
122 2005) and dogs (McClain *et al.* 2006). With the exception of one cat diagnosed with  
123 subacute suppurative endometritis (discussed below), no histological abnormalities were  
124 detected in ovarian or uterine tissue from any other cat, and no histological differences  
125 were detected between treatment and control groups. The number of corpora lutea, or  
126 primary, secondary, tertiary, mature or atretic follicles in cat ovaries did not differ  
127 between groups. It is possible that the lower dose provided (reflecting the higher end of  
128 the typical dietary intake range calculated by Bell *et al.*, 2006), and the use of an oral  
129 administration route, which results in low bioavailability (Cave *et al.*, 2007), may explain  
130 the differences observed here compared to previous studies, whereby only dogs exposed  
131 to 500 mg/kg BW (compared to 50 mg/kg BW and 150 mg/kg BW groups) exhibited  
132 uterine hypertrophy (McClain *et al.*, 2006). Alternatively, the duration of administration  
133 in this present study exceeds previous studies, and acute responses may have been  
134 missed. This is supported by the finding that uterine hypertrophy was detected in dogs

135 following 13 weeks of exposure to a high genistein dose (500 mg/kg BW) but not  
136 following 52 weeks exposure (McClain *et al.*, 2006)

137 However, differences detected in receptor staining between groups indicate a role for  
138 dietary isoflavones in modulating reproductive physiology in domestic cats. Expression  
139 of ER $\alpha$  and ER $\beta$  receptors was greater in treated cats compared to control cats ( $P < 0.05$ ),  
140 with the exception of ER $\alpha$  in the uterine basal endometrium and myometrium, where  
141 expression was greater in control tissues ( $P < 0.05$ ; Table 2). No difference was detected  
142 between groups in ER $\beta$  or PR expression in the myometrium. The up-regulation of  
143 cellular expression of ER $\alpha$  and ER $\beta$  in the ovarian cortex, medulla and uterine  
144 endometrium observed in response to isoflavone treatment here, has been reported in  
145 other species (Jefferson *et al* 2002; Chrzan and Bradford 2007). Genistein and daidzein  
146 are capable of binding to both ERs, with preferential binding and transactivation shown  
147 for ER $\beta$  (reviewed in Rietjens *et al* 2013). These isoflavones act as nuclear receptor  
148 ligands to enhance interactions between oestrogen-related receptors and proline-rich  
149 nuclear receptor coactivator (PNRC) (reviewed in Ricketts *et al.*, 2005).

150 **Table 2 here**

151 Conversely, proportional expression of the PR was typically down-regulated in  
152 isoflavone-treated cat uterine and ovarian sections. Progesterone receptor expression was  
153 lower in treatment cats compared to control cats in ovarian cortex and uterine apical  
154 endometrium ( $P < 0.05$ ), while no difference was detectable in the ovarian medulla and  
155 myometrium; in the uterine basal endometrium PR expression was greater in treatment  
156 cats ( $P < 0.05$ ). These sex steroid receptors are important mediators in the control of  
157 oestrogen- and progesterone-induced effects during oestrous cyclicity and pregnancy,

158 such that modifications at the receptor level may be reflected in aberrant physiological  
159 responses or fertility.

160 One cat in the treatment group was diagnosed with subacute suppurative endometritis at  
161 267 days of age. This cat's tissue morphological data was excluded due to its diseased  
162 state. The development of suppurative endometritis in an isoflavone-treated cat was an  
163 unexpected finding. This condition is not common in cats, and is generally only reported  
164 in cats older than eight years of age (Agudelo 2005), but further research is required to  
165 determine the role that isoflavones may have played in the onset of the condition in the  
166 cat reported here.

## 167 **Conclusions**

168 Preliminary findings suggest that the isoflavones, genistein and daidzein may exert  
169 modulatory effects on the expression of sex steroid receptors in feline uterine tissue.  
170 Given the limited sample size our findings should be considered as preliminary and  
171 interpreted with caution. Future investigation should include life-time evaluation of  
172 feline fertility and fecundity, with increased sample sizes.

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**Table 1** Wet weights (as grams or % body weight) of reproductive tracts obtained from domestic cats following chronic isoflavone exposure (treatment group) or control animals<sup>1,2</sup>.

	Control group mean (SD)		Treatment group mean (SD)	
	g	% BW	g	% BW
Entire tract wet weight	1.99 (0.41)	0.07 (0.01)	2.15 (0.45)	0.07 (0.01)
Left ovary	0.17 (0.15)	0.01 (0.00)	0.13 (0.01)	0.004 (0.00)
Right ovary	0.12 (0.2)	0.004 (0.00)	0.11 (0.03)	0.004 (0.00)

<sup>1</sup>Values are expressed as mean ( $\pm$  SD). No significant differences were detected between groups ( $p > 0.05$ ).

<sup>2</sup> Data from eight control cats and three treatment cats (one treatment cat was diagnosed with acute suppurative endometritis and as such the diseased state of her reproductive tract rendered it unsuitable for inclusion in this dataset)

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**Table 2** The proportion of cells staining positive for ER $\alpha$ , ER $\beta$ , or PR in the reproductive tract of cats exposed to dietary genistein and daidzein, compared to control cats<sup>1</sup>.

	ER $\alpha$		ER $\beta$		PR	
	Control	Treatment	Control	Treatment	Control	Treatment
Ovarian cortex	0.58 (0.44) <sup>a</sup>	0.79 (0.18) <sup>b</sup>	0.57 (0.46) <sup>a</sup>	0.83 (0.26) <sup>b</sup>	0.75 (0.49) <sup>b</sup>	0.66 (0.31) <sup>a</sup>
Ovarian medulla	0.52 (0.46) <sup>a</sup>	0.94 (0.05) <sup>b</sup>	0.79 (0.21) <sup>a</sup>	0.93 (0.13) <sup>b</sup>	0.72 (0.29) <sup>a</sup>	0.76 (0.42) <sup>a</sup>
Uterine apical endometrium	0.91 (0.13) <sup>a</sup>	0.95 (0.09) <sup>b</sup>	0.63 (0.39) <sup>a</sup>	0.88 (0.11) <sup>b</sup>	0.81 (0.30) <sup>b</sup>	0.64 (0.44) <sup>a</sup>
Uterine basal endometrium	0.91 (0.22) <sup>b</sup>	0.84 (0.19) <sup>a</sup>	0.79 (0.33) <sup>a</sup>	0.95 (0.11) <sup>b</sup>	0.76 (0.20) <sup>a</sup>	0.88 (0.22) <sup>b</sup>
Myometrium	0.60 (0.33) <sup>b</sup>	0.32 (0.17) <sup>a</sup>	0.53 (0.35) <sup>a</sup>	0.59 (0.22) <sup>a</sup>	0.64 (0.31) <sup>a</sup>	0.63 (0.12) <sup>a</sup>

<sup>1</sup>Standard deviation is shown in parentheses. Values with different superscripts (within row for each respective sex steroid receptor) are significantly different ( $p < 0.05$ ).