

Non-invasive monitoring of stoat reproductive hormones

Frank Molina, Susana La Falci, Vaughan Myers and Duncan McLane

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Cover: Stoat (*Mustela erminea*) from the Landcare Research captive colony.
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CONTENTS

Abstract	5
1. Introduction	6
2. Objectives	7
3. Methods	7
3.1 Faecal hormone extractions	8
3.2 High performance liquid chromatography	8
3.3 Enzyme immunoassays	9
3.3.1 Laboratory validation	10
3.3.2 Biological validation	10
3.4 Longitudinal hormone profiles	11
3.5 Photoperiod treatment to advance seasonal reproduction	12
4. Results	12
4.1 Validation of enzyme immunoassays	12
4.1.1 Faecal hormone extractions	12
4.1.2 High performance liquid chromatography	12
4.1.3 Laboratory validation	14
4.1.4 Biological validation	15
4.2 Longitudinal hormone profiles	16
4.3 Photoperiod treatment to advance seasonal reproduction	19
5. Discussion	20
6. Conclusions	22
7. Recommendations	22
8. Acknowledgements	23
9. References	23

Non-invasive monitoring of stoat reproductive hormones

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ABSTRACT

This study reports the development of faecal hormone assays to non-invasively monitor the major hormones of reproduction in captive stoats (*Mustela erminea*). A procedure was developed for extracting testosterone, oestradiol and progesterone metabolites from stoat faeces. High performance liquid chromatography analysis revealed that testosterone remains in its native form, whereas oestradiol and progesterone are metabolised before excretion in stoat faeces. Enzyme-immunoassays were validated to monitor male (testicular) and female (ovarian) function, based on changes in testosterone, oestradiol and progesterone metabolite concentrations in stoat faeces after hormone challenge and/or gonadectomy. Circannual hormone profiles were produced entirely non-invasively in the stoat. In males, faecal testosterone excretion was found to be seasonal, with levels being higher during the breeding season (September–January) than outside it (February–August). In females, increases in faecal oestradiol were found to correspond with oestrus. Pregnancy was confirmed in stoats before the birth of kits based on increases in concentrations of faecal progesterone metabolites associated with implantation. However, with the limited samples available, neither the oestradiol nor progesterone metabolite assay could detect differences between pregnant and non-pregnant females during diapause and before implantation. Radio-immunoassays of faecal testosterone supported other investigations, which found that photoperiod treatment advanced seasonal reproduction in stoats. Non-invasive reproductive hormone monitoring will enhance our ability to monitor captive breeding and manipulation of stoat reproduction. In the future, this will be a valuable tool to add to established protocols for testing fertility controls targeting reproductive function in stoats.

Keywords: stoat, *Mustela erminea*, reproduction, faecal hormone metabolites, enzyme immunoassays, seasonality, photoperiod, New Zealand

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1. Introduction

Stoats (*Mustela erminea*) are one of a suite of predators that have been introduced to New Zealand and are responsible for the decline of several native iconic bird species such as kiwi (*Apteryx* spp.) and kaka (*Nestor meridionalis*) (see Parkes & Murphy 2004). Current control techniques, including labour-intensive trapping and ground-based non-specific poisoning, are inadequate, and raise concerns about non-target impacts, humaneness and environmental risk. Fertility control is one approach being evaluated as a sustainable and potentially species-specific method of stoat control for use over large areas with enhanced acceptability and safety (Norbury 2000). In combination with current methods, it would offer a cost-effective solution for managing stoat overabundance in the long term (Barlow & Choquenot 2002).

Stoats have an unusual breeding system that lends itself to disruption at various stages. This includes egg development and fertilisation in spring, followed by 8–9 months of arrested embryonic development (diapause), and then a 4-week period of post-implantation pregnancy before birth the following spring. Newborn males become sexually mature by the following breeding season. However, female stoats (both adults and juveniles as young as 3 weeks old) have the capacity to mate and produce a large litter of young each year, meaning that the disruption of stoat reproduction has the potential to dramatically increase the effectiveness of control (Barlow & Choquenot 2002). Several agents that have the potential to disrupt stoat reproduction have been identified (e.g. chemicals, proteins, peptides and antigens; Hinds et al. 2000), and some have recently been tested in stoats (e.g. zona pellucida antigens; Dall & Duckworth 2005) or related mustelids (e.g. pimozide, a dopamine antagonist that terminates diapause in mink (*Mustela vison*); Marks et al. 2006).

To test the effects of these candidate agents on fertility, it is crucial to have reproductively functioning stoats in captivity. Our group has successfully bred stoats in captivity (O'Connor et al. 2006) and in the present study we have used this colony to investigate non-invasive monitoring of stoat reproductive hormones. The work focused on measuring levels of the following hormones in faeces: testosterone to monitor male reproductive function, oestradiol metabolites to monitor female reproductive cycles, and progesterone metabolites to monitor pregnancy. This non-invasive approach is far superior to traditional blood sampling because collecting multiple blood samples is not feasible in stoats and there is evidence in other carnivores that the stress of handling and anaesthesia can alter the normal pattern of hormonal secretion (Young et al. 2001, 2004). This research aimed to broaden our knowledge of the reproductive hormone profiles in stoats during natural and manipulated breeding cycles. Reproductive hormone monitoring will be a valuable tool if fertility control targeting stoat reproductive function is contemplated in the future.

2. Objectives

The objective of this study was to develop faecal hormone assays to non-invasively monitor the major hormones of reproduction in both male and female stoats so that benchmark values for natural cycles and the effect of artificial manipulation could be determined by:

- Developing procedures for extracting reproductive hormone (testosterone, oestradiol and progesterone) metabolites from stoat faecal samples.
- Undertaking high performance liquid chromatography (HPLC) analyses to determine which enzyme immunoassays (EIAs) are suitable for measuring reproductive hormone metabolites from stoat faecal extracts.
- Establishing laboratory and biological validation of selected EIAs for monitoring male (testicular) and female (ovarian) reproductive function from stoat faecal extracts.
- Producing faecal hormone profiles in relation to reproductive status of male and female stoats both during and outside the breeding season.
- Producing faecal reproductive hormone profiles of photoperiod-advanced stoats from an earlier study (O'Connor et al. 2006).

3. Methods

All stoats were maintained at the Animal Facility at Landcare Research, Lincoln, and had undergone at least an 8-week period of acclimatisation to captivity. Male and female stoats were housed in individual cages (60 × 150 × 90 cm high) under an outside shelter. Each stoat was assigned an identification number and provided with a nest box (40 × 33 × 15 cm high) containing shredded paper as nesting material. Females in the Captive Stoat Breeding Facility were housed in individual outdoor pens (2 × 4 × 2 m high) with natural cover and shelter and supplied with two nest boxes containing shredded paper and feathers as nesting material. Stoats were fed a rotation of dead day-old chicks, chicken pet mince and mutton/beef pet mince in the afternoon, and were provided with water *ad libitum*.

Sample collection occurred concurrently with routine husbandry and/or as part of experimental manipulations as specifically detailed below. Faecal material was collected from nest boxes of individual stoats within 24 h of excretion, and was placed in labelled 1.5-mL polypropylene Eppendorf tubes and stored at -20°C until extraction of reproductive hormone metabolites.

All research involving stoats was performed in accordance with the Animal Welfare Act 1999 and approved by the Animal Ethics Committee of Landcare Research, Lincoln (approval numbers 00/9/7 and 04/06/01).

3.1 FAECAL HORMONE EXTRACTIONS

Methods that have been used for extracting reproductive hormone metabolites from faecal samples of other domestic and non-domestic species (Brown et al. 2003) were unsuitable for stoats, due to the large proportion of feathers in faecal samples. A series of pilot studies was undertaken to test several extraction techniques including adding trichloroacetic acid to precipitate keratin; using ethanol v. methanol as solvents; boiling v. centrifugation of extracts; and using silica gel v. filters to eliminate feathers. Ultimately, samples were extracted after modification of a procedure that has been used for black-footed ferrets, *Mustela nigripes* (Brown 1997). Briefly, samples were lyophilised, pulverised and sieved through a fine mesh filter to remove feathers. Aliquots of well-mixed powder (30 mg) were boiled in 5 mL of aqueous ethanol (90%) for 20 min. Extracts were centrifuged at 620g for 20 min and the supernatant recovered. The pellet was resuspended in 5 mL of 90% ethanol, vortexed for 1 min and then recentrifuged and the supernatant recovered. Both ethanol supernatants were pooled and evaporated to dryness under air in labelled glass tubes (12 × 75 mm). Extracts were then dissolved in 1 mL methanol, sonicated for 5 min, vortexed for 15 s, covered with parafilm and stored at -20°C.

To verify the effectiveness of the procedure, extraction efficiency was determined by adding a known concentration of ³H-labelled testosterone, oestrogen, or progesterone tracer to faecal material before extraction. Extraction efficiency was also determined using unlabelled hormone. Standards used in each EIA (12 ng testosterone, 5 ng oestrone glucuronide, or 80 ng progesterone) were added to samples of faecal material and then extracted as detailed above. These extracts were diluted (1:16 and 1:32 for testosterone, 1:8 for oestradiol metabolites, and 1:160 for progesterone metabolites) in assay buffer (0.1 mol/L sodium phosphate buffer, 0.149 mol/L NaCl, 0.1% bovine serum albumin, pH 7.0) and run on the appropriate EIA (detailed in section 3.3). Extraction efficiency was calculated as the amount of hormone observed relative to the amount expected, and expressed as a percentage (mean ± SEM).

3.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The number and types of reproductive hormone metabolites in male and female stoat faecal extracts were determined by reverse-phase HPLC (Microsorb C-18 Column, particle size diameter of 5 µm). Pooled extracts of faecal samples (*n* = 6) that were likely to contain high concentrations of testosterone (from males during the breeding season), oestradiol (from oestrous females), and progesterone (from pregnant females) metabolites were evaporated to dryness under air and sent to the Conservation and Research Center, National Zoological Park, Smithsonian Institution, Front Royal, Virginia, USA. Before HPLC, extracts were reconstituted in 0.5 mL phosphate-buffered saline (PBS) at pH 5, passed through a C-18 matrix column, and eluted with 5 mL methanol to remove contaminants. Filtered extracts were evaporated to dryness under air and reconstituted in methanol.

For separation of testosterone metabolites, filtered extracts were eluted (333- μ L fractions; 1 mL/min flow rate) using an isocratic (constant) gradient of 45% acetonitrile:water over 30 min. Oestradiol metabolites were separated using a gradient of 20% to 80% methanol over 120 min (1-mL fractions; 1 mL/min flow rate). Progesterone metabolites were separated using a gradient of 20% to 80% acetonitrile:water over 120 min (1-mL fractions; 1 mL/min flow rate). Faecal eluates were taken to dryness, reconstituted in assay buffer, and quantified for immunoactivity using various EIAs that are routinely used at the Smithsonian Institution. The elution position of radiolabelled steroids (^3H -testosterone, ^3H -oestrone, ^3H -oestrone sulphate, ^{14}C -oestradiol, and ^{14}C -progesterone) that had been added to faecal extracts prior to the relevant HPLC run were also determined and expressed as radioactive disintegrations per minute (dpm). The profiles produced were graphed together and used to determine whether stoat reproductive hormones were excreted as native or metabolised forms and, hence, to identify the EIAs most suitable for monitoring testicular and ovarian activity from faecal samples.

3.3 ENZYME IMMUNOASSAYS

Reproductive hormone metabolite concentrations in stoat faecal extracts were quantified by EIA using procedures based on those described for other mammals (Munro & Stabenfeldt 1984; Munro & Lasley 1988; Munro et al. 1991). Concentrations of faecal testosterone were determined using a polyclonal anti-testosterone antiserum (R156/7) diluted 1:15 000, horseradish peroxidase (HRP)-conjugated testosterone label diluted 1:15 000 and testosterone standards (2.34–600 pg/well). Oestradiol metabolites were determined using a polyclonal anti-oestrone conjugate antiserum (R522-2) diluted 1:40 000, HRP-conjugated oestrone glucuronide label diluted 1:25 000, and oestrone glucuronide standards (0.78–200 pg/well). Concentrations of faecal progesterone metabolites were determined using a monoclonal anti-progesterone (Quidel Corporation, San Diego, USA) antiserum (CL425) diluted 1:20 000, HRP-conjugated progesterone 3-O-carboxymethyloxime label diluted 1:35 000, and progesterone standards (0.78–200 pg/well). Antisera and HRP ligands were all provided by Coralie Munro (University of California, Davis, California, USA). Antisera cross-reactivity for the EIAs have been reported previously (testosterone: deCatanzaro et al. 2003; oestrone conjugate: Munro et al. 1991; progesterone: Graham et al. 2001).

The assays were carried out on Nunc MaxiSorpTM plates that were coated with 50 μ L of antibody diluted to the appropriate concentration in a coating buffer (50 mmol/L bicarbonate buffer, pH 9.6) and incubated for at least 12 h at 4°C. Plates were washed with phosphate-buffered saline containing 0.5 mL/L Tween 20 to rinse away any unbound antibody. Stocks of standards, high- and low-binding internal controls, faecal extracts¹, and HRP labels were diluted to the appropriate concentration in assay buffer. For the testosterone

¹ Extracts stored in methanol were diluted directly with assay buffer if the dilution rate was greater than 1:10. At lower dilution rates, and to mitigate interference of methanol on plate binding, aliquots of the extracts reconstituted in methanol were taken to dryness and reconstituted in assay buffer, sonicated for 5 min, and vortexed for 15 s.

and progesterone EIAs, 50 µL of standard, internal control or faecal extract was added to each well. For the oestrone conjugate EIA, 50 µL of assay buffer was added to each well immediately after washing and incubated at room temperature for 2-5 h before the addition of 20 µL of standard, internal control or faecal extract. For all assays, 50 µL of the corresponding HRP label was then added to each well and the plates were incubated at room temperature for 2 h. Plates were washed and 50 µL of a substrate solution (citrate buffer, H₂O₂ and tetramethylbenzidine) was added to each well. Stopping solution (50 µL of 0.5 mol/L H₂SO₄) was added based on visual inspection of plates, so that the optical density of the zero wells would read between 0.7 and 1 (usually after 7-10 min incubation at room temperature).

Plates were read at 450 nm (reference 630 nm) on a microplate reader. Blank absorbance was subtracted from each reading to account for non-specific binding. In all assays, standard curves were generated and a regression line fitted; the equation was then used to determine the concentration of reproductive hormone metabolites in faecal samples. All data are expressed on a dry-faecal-weight basis.

3.3.1 Laboratory validation

Laboratory validation of assays was achieved by demonstrating:

- Parallelism between serial two-fold dilutions of pooled faecal extracts (neat, 1:2, 1:4, 1:8, 1:16, etc. to 1:4096) and the respective standard curves.
- Significant recovery of exogenous steroid standard added to faecal extracts.

Recovery was expressed as a linear regression formula ($y = mx + b$, where y = amount observed, x = amount of hormone expected, and m = slope of the line) and the multiple correlation coefficient was squared to produce the coefficient of determination (R^2). Slopes greater or less than 1 represent an over- or under-estimation of hormone mass, respectively. For all experiments, parallel displacement curves were used to determine the dilution rates of individual faecal extracts before assay and were based on the concentration of pooled faecal extracts that resulted in 50% binding. Assay sensitivity was calculated as the value 2 standard deviations from the mean response of the blank (zero binding) samples, and expressed as a mean \pm SEM. Intra- (within) and inter- (between) assay coefficients of variation were determined from high- (approximately 70%) and low- (approximately 30%) binding internal controls run on all assays.

3.3.2 Biological validation

Biological validation of the assays was achieved by performing a hormone challenge of male and female stoats and gonadectomy of male stoats to demonstrate a relationship between treatment and the subsequent excretion of the target hormone (Brown et al. 2003). During August 2004, male stoats 223 and 360, and female ($n = 4$) stoats were treated with a single subcutaneous injection of 400 IU human chorionic gonadotrophin (hCG, Chorulon, Intervet). Daily faecal samples were collected 7 days before and up to 7 days after treatment. Faecal extracts were diluted 1:40, 1:12.5 and

1:40 in assay buffer before quantification of testosterone, oestradiol and progesterone metabolites, respectively, using the relevant EIA as detailed above. In addition, male stoats 360 and 361 were gonadectomised during November 2004 and 15 days later were treated with a single subcutaneous injection of 30 µg/kg testosterone. Faecal samples were collected from up to 7 days before through to 7 days after hormone challenge. Pre-gonadectomy samples were only collected from male stoat 361 from up to 4 days before gonadectomy. Extracts were diluted 1:8 in assay buffer before quantification of faecal testosterone by EIA.

3.4 LONGITUDINAL HORMONE PROFILES

Longitudinal assessment of faecal testosterone concentrations from male stoats ($n=4$) that had been maintained in captivity and exposed to normal photoperiod was undertaken to evaluate testicular activity. To cover the periods during (September through to January) and outside (February through to August) the natural breeding season observed in our captive colony (O'Connor et al. 2006), faecal samples were collected fortnightly over a 13-month period from October 2001 to the end of October 2002. Extracts were diluted 1:20 in assay buffer before quantification of faecal testosterone by EIA.

To evaluate ovarian activity, female stoats that had been exposed to normal photoperiod were assessed for faecal oestradiol and progesterone metabolites by EIA. Faecal samples were collected from wild-caught females used in our captive breeding colony from late October 2001 (or from late March 2002 in the case of female stoat 193) through to early November 2002. This was undertaken to cover the entire reproductive period from oestrus detection (vulval swelling), to successful mating (absence of swollen vulva after male removed), presumptive pregnancy (including diapause and implantation) and birth (O'Connor et al. 2006). Data are presented from four females thought to be pregnant, including females 112 and 193 that gave birth, and females 115 and 195 that did not. During the 2003/04 season, previously captive-born females were used for breeding stoats in captivity. Faecal samples were collected from late June through to early October 2004 to investigate the period of late diapause and implantation in more detail. Data are presented from females 356 and 357 that gave birth, and females 354 and 358 that were not pregnant (came into oestrus but were not successfully mated). Extracts from all females were diluted 1:8 in assay buffer before quantification of faecal oestradiol metabolites using the oestrone conjugate EIA. The same diluted extracts were diluted a further five-fold in assay buffer (final dilution 1:40) before quantification of faecal progesterone metabolites using the progesterone EIA.

3.5 PHOTOPERIOD TREATMENT TO ADVANCE SEASONAL REPRODUCTION

The exposure of stoats to a simulated summer day length in winter was reported to advance seasonal reproduction significantly in both male and female stoats, and result in out-of-season mating (O'Connor et al. 2006). To correlate these findings with reproductive hormone profiles, faecal hormone monitoring of control and photoperiod-advanced stoats was undertaken (McLane 2004).

From May 2003, adult male ($n=6$) and female ($n=6$) stoats were exposed to increased durations of artificial lighting, which reached 16 h/day on 30 June and continued at this duration until November. Controls ($n=6$ male and $n=6$ female stoats) were exposed to normal photoperiod. Testis size was monitored in males and vaginal cytology and physical changes associated with oestrus were monitored in females (O'Connor et al. 2006). Faecal samples were collected, stored and extracted before analysis, as described at the start of section 3. Quantification of faecal testosterone and oestradiol was undertaken using radioimmunoassay (RIA) techniques validated for use in stoats (McLane 2004), as the EIAs were still in development at the time. Faecal extracts were diluted 1:4 in assay buffer before analysis. All data are expressed on a dry-faecal-weight basis.

4. Results

4.1 VALIDATION OF ENZYME IMMUNOASSAYS

4.1.1 Faecal hormone extractions

Extraction efficiencies ranging from 70% to 90% were obtained reliably after the addition of ^3H -labelled testosterone, oestrogen or progesterone tracer before extraction. Extraction efficiencies of $98.8 \pm 3.1\%$, $68.7 \pm 5.3\%$ and $89.0 \pm 4.7\%$ were also obtained after the addition of unlabelled testosterone, oestrone glucuronide and progesterone, respectively, to each of six faecal samples before extraction, and quantification on the corresponding EIA.

4.1.2 High performance liquid chromatography

The evaluation of faecal extracts from male stoats by HPLC revealed that the immunoactivity measured by the testosterone EIA was almost entirely associated with native unconjugated testosterone (Fig. 1). In contrast, we identified multiple immunoreactive substances in HPLC eluates of faecal extracts from female stoats using various EIAs that cross-react with native and metabolised forms of oestradiol and progesterone. Using an oestradiol EIA, native oestradiol was found to be excreted in stoat faeces but the overall mass recovery was low (data not shown). When using the oestrone conjugate EIA, immunoactivity was associated with oestrone and a conjugated form of oestrogen, possibly oestradiol glucuronide or oestrone glucuronide, but not oestrone sulphate (Fig. 2). Three major immunoreactive peaks were found using a progesterone EIA but none of the immunoactivity was associated with native progesterone (Fig. 3) or pregnanediol-3-glucuronide, a conjugated form of progesterone, when using an EIA that cross-reacted with this compound (data not shown).

Figure 1. HPLC separation of extracted faecal samples from male stoats (*Mustela erminea*). Immunoactivity of each fraction was determined by testosterone EIA using R156/7 antibody. Retention times of immunoactive peaks were compared with radiolabelled testosterone reference tracer.

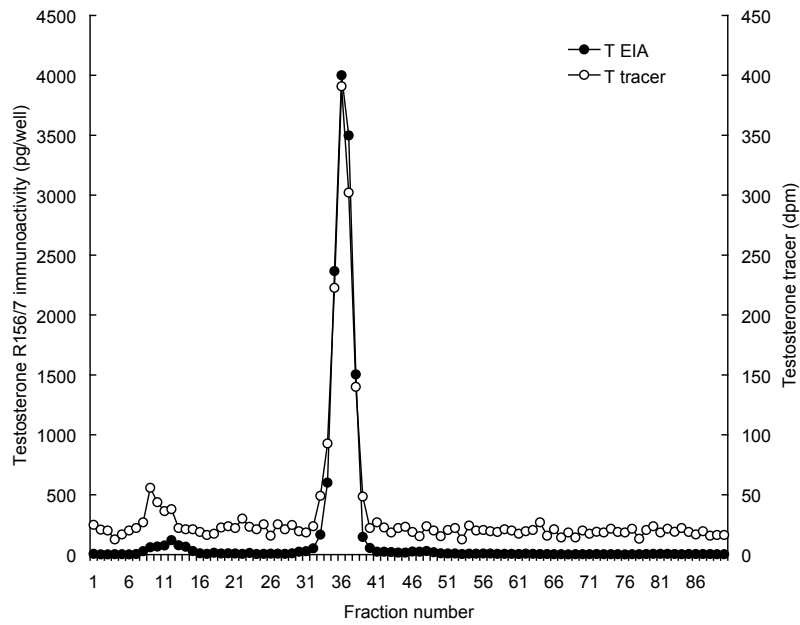


Figure 2. HPLC separation of extracted faecal samples from female stoats (*Mustela erminea*). Immunoactivity of each fraction was determined by oestrone conjugate EIA using R522-2 antibody. Retention times of immunoactive peaks were compared with radiolabelled oestrone, oestrone sulphate, and oestradiol reference tracers.

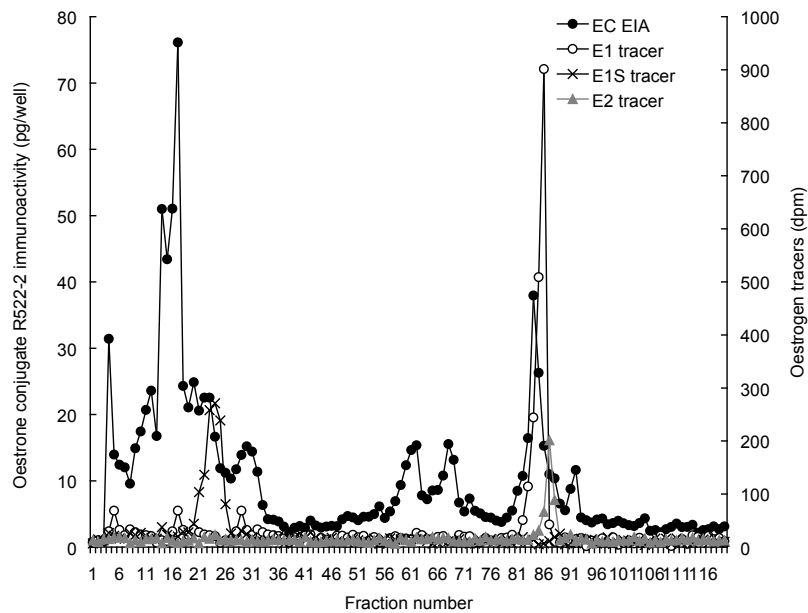
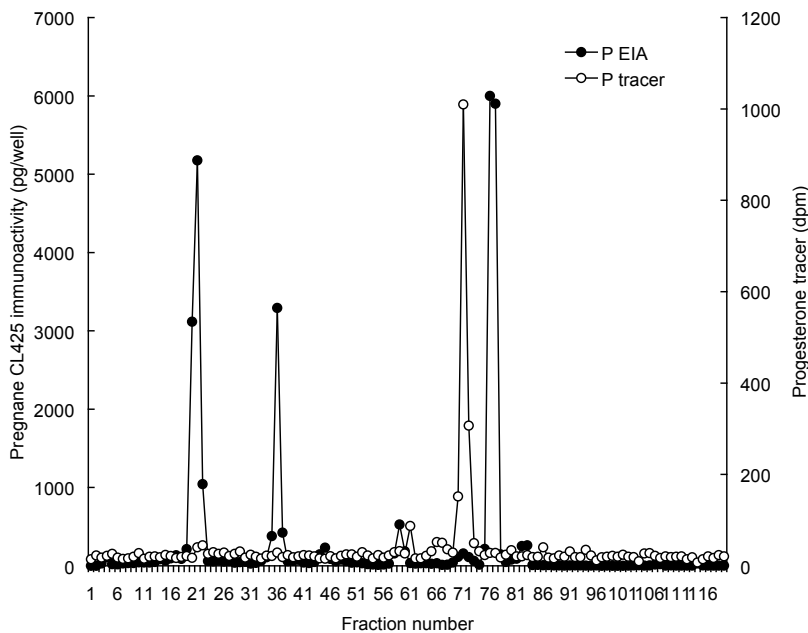


Figure 3. HPLC separation of extracted faecal samples from female stoats (*Mustela erminea*). Immunoactivity of each fraction was determined by progesterone EIA using CL425 antibody. Retention times of immunoactive peaks were compared with radiolabelled progesterone reference tracer.



4.1.3 Laboratory validation

The validity of the testosterone EIA was confirmed by serial dilutions of pooled faecal extracts from male stoats that produced displacement curves parallel to those of testosterone standards (Fig. 4A-C), and significant recovery of exogenous testosterone (4.69–150 pg; $y = 0.97x + 2.58$, $R^2 = 0.999$; $n = 4$) added to faecal extracts. The sensitivity of the testosterone EIA was 2.1 ± 0.3 pg/well ($n = 13$). The intra- and inter-assay coefficients of variation were 3.2% and 7.6% for the high-binding internal control, and 2.7% and 15.3% for the low-binding internal control, respectively ($n = 12$).

The validity of the oestrone conjugate EIA was confirmed by serial dilutions of pooled faecal extracts from female stoats that produced displacement curves parallel to those of oestrone glucuronide standards (Fig. 5A & B), and significant recovery of exogenous oestrone glucuronide (1.56–50 pg; $y = 1.09x - 1.20$, $R^2 = 0.998$; $n = 4$) added to faecal extracts. Sensitivity of the oestrone conjugate EIA was 1.9 ± 0.2 pg/well ($n = 12$). The intra- and inter-assay coefficients of variation were 3.1% and 5.6% for the high-binding internal control, and 5.4% and 6.7% for the low-binding internal control, respectively ($n = 11$).

The validity of the progesterone EIA was confirmed by serial dilutions of pooled faecal extracts from male stoats that produced displacement curves parallel to those of progesterone standards (Fig. 6A & B), and significant recovery of exogenous progesterone (1.56–50 pg; $y = 0.93x + 0.43$, $R^2 = 0.995$; $n = 4$) added to faecal extracts. Sensitivity of the progesterone EIA was 3.2 ± 0.5 pg/well ($n = 12$). The intra- and interassay coefficients of variation were 3.6% and 10.0% for the high-binding internal control, and 4.1% and 16.6% for the low-binding internal control, respectively ($n = 11$).

Figure 4. Binding displacement curves following serial dilution and testosterone EIA of pooled faecal extracts and the appropriate standard from male stoats (*Mustela erminea*): A. hCG challenged (HC); B. gonadectomised then testosterone challenged (GTC); C. exposed to normal photoperiod and used in the longitudinal study of seasonality (S). Arrows indicate the concentration at which approximately 50% binding was observed.

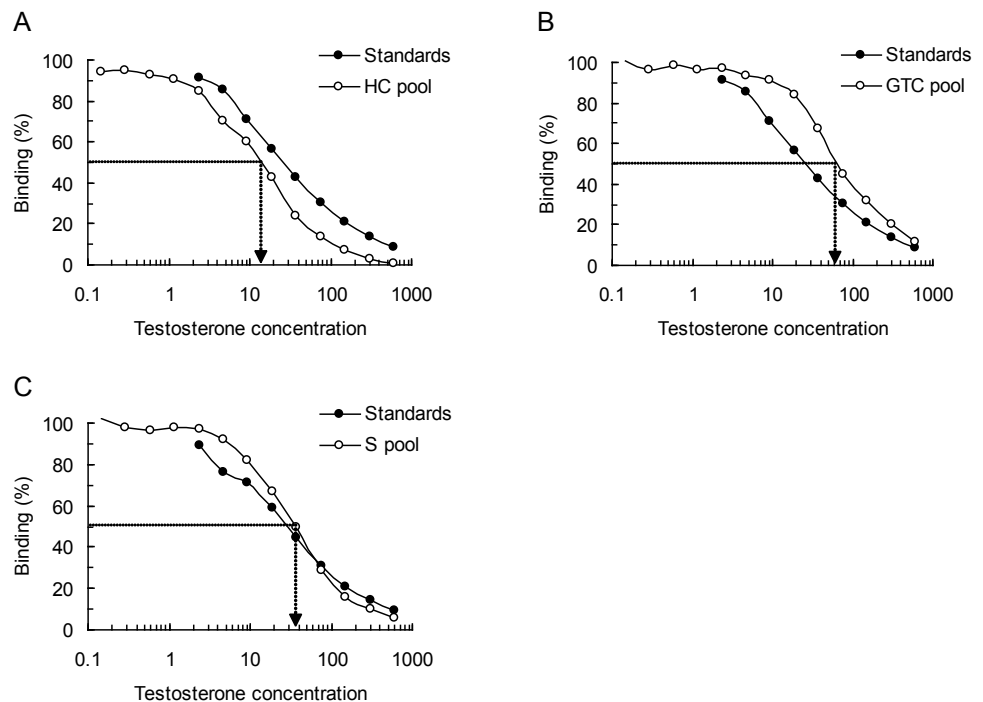


Figure 5. Binding displacement curves following serial dilution and oestrone conjugate EIA of pooled faecal extracts and the appropriate standard from female stoats (*Mustela erminea*): A. hCG challenged (HC); and B. in the captive breeding (CB) colony. Arrows indicate the concentration at which approximately 50% binding was observed.

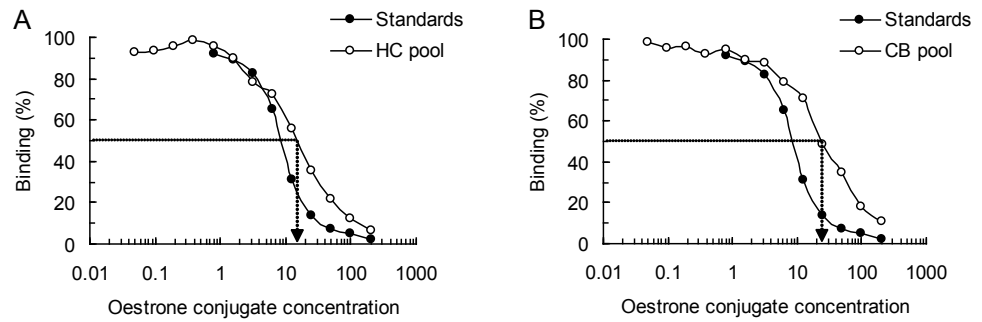
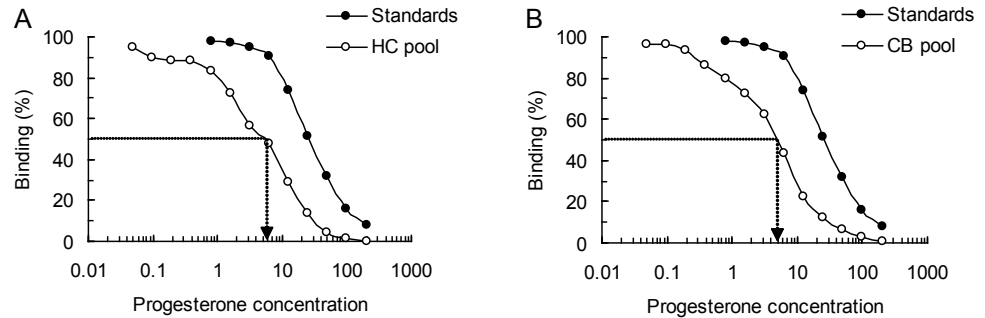


Figure 6. Binding displacement curves following serial dilution and progesterone EIA of pooled faecal extracts and the appropriate standard from female stoats (*Mustela erminea*): A. hCG challenged (HC); and B. in the captive breeding (CB) colony. Arrows indicate the concentration at which approximately 50% binding was observed.



4.1.4 Biological validation

Male stoats 223 and 360 responded physiologically to hCG challenge (Fig. 7A), with an 844% and 293% increase in mean faecal testosterone concentrations above baseline levels, respectively (male 223: pre-hCG = 233.6 ± 29.7 ng/g, post-hCG = 1970.5 ± 512.8 ng/g, Mann Whitney test, $U=0.0$, $df=5,6$, $P=0.004$; male 360: pre-hCG = 228.8 ± 23.0 ng/g, post-hCG = 670.7 ± 164.0 ng/g, Mann Whitney test, $U=2.0$, $df=5,6$, $P=0.017$). In contrast, gonadectomy of male 361 resulted in a 79% decrease in mean faecal testosterone concentration from 476.0 ± 135.7 ng/g to 99.6 ± 25.8 ng/g (Mann Whitney test, $U=0.0$, $df=5,3$, $P=0.036$; Fig. 7B). However, subsequent testosterone treatment of this male and another gonadectomised stoat (male 360) did not alter the faecal testosterone excretion pattern significantly. The $30 \mu\text{g}/\text{kg}$ dose of testosterone used for this challenge may have been too low or the change too transient to be detected.

For female stoats ($n=4$), the mean faecal oestradiol metabolite concentration after hCG treatment was 320% above baseline levels (pre-hCG = 51.1 ± 16.6 ng/g, post-hCG = 163.3 ± 29.7 ng/g, paired sample t -test, $t=5.04$, $df=3$, $P=0.015$; Fig. 8A). There was an effect of sampling period on mean progesterone metabolite concentrations (ANOVA, $F=7.47$, $df=2,5$, $P=0.032$). The day after challenge, progesterone metabolite concentration (1258.8 ± 414.5 ng/g) peaked at 197% above baseline levels (639.8 ± 124.2 ng/g) before returning to pre-treatment levels thereafter (568.9 ± 76.4 ng/g; Fig. 8B).

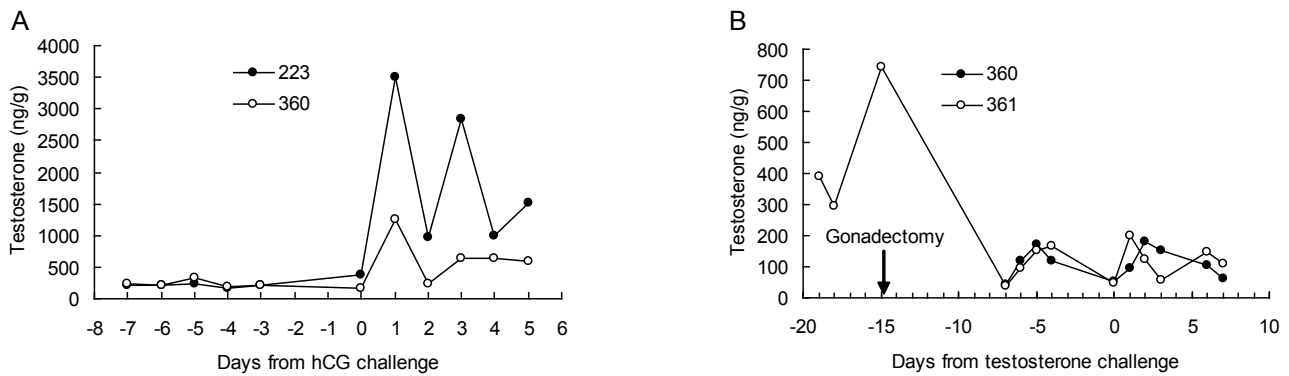


Figure 7. Faecal testosterone concentrations after hormone challenge of male stoats (*Mustela erminea*): A. stoats 223 and 360 challenged with hCG; and B. stoats 360 and 361 that were gonadectomised 15 days before being challenged with testosterone.

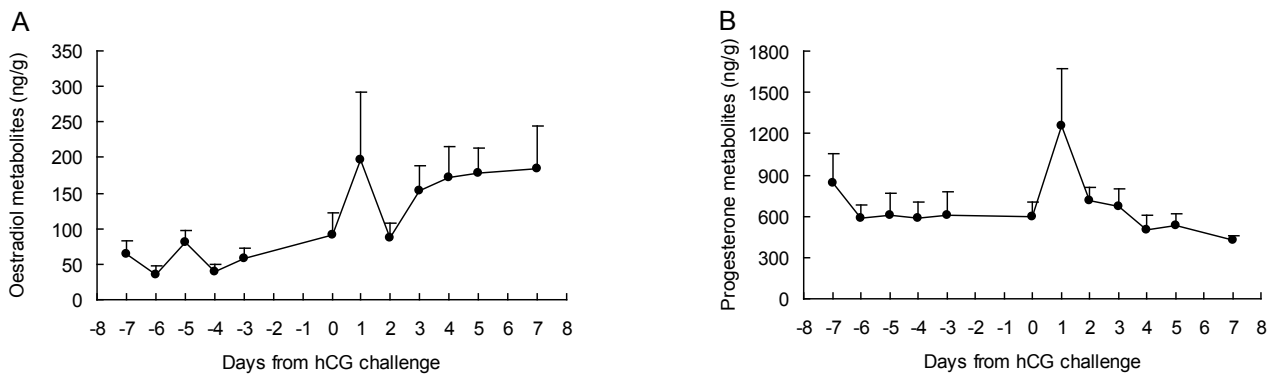


Figure 8. Mean + SEM faecal hormone concentrations after hCG challenge of female stoats (*Mustela erminea*; $n = 4$): A. oestradiol metabolites; and B. progesterone metabolites.

4.2 LONGITUDINAL HORMONE PROFILES

The profile of mean (\pm SEM) faecal testosterone for four captive male stoats that were exposed to normal photoperiod over a 13-month period is shown in Fig. 9. There was a significant effect of season on faecal testosterone excretion (paired sample t -test, $t = 4.21$, $df = 3$, $P = 0.024$), with mean concentrations being lowest outside the breeding season (February through to August; 203.0 ± 17.6 ng/g) and highest during the breeding season (September through to January; 418.8 ± 68.3 ng/g).

Individual profiles of oestradiol and progesterone metabolites for captive female stoats exposed to normal photoperiod are shown in Figs 10 and 11. Vulval swelling, as an external sign of oestrus, was detected in females 112, 115 and 195 between November 2001 through to January 2002, and this corresponds with peaks observed in faecal oestradiol metabolites over the same time period (> 250 ng/g; Fig. 10A, C & D). Female 112 in particular was noted to have successfully mated in mid-December 2001, as evidenced by the absence of vulval swelling after removal of a male stoat, and this appears to be reflected in the drop in faecal oestradiol metabolites and the concomitant rise in progesterone metabolites (Fig. 10A). Stoat kits were born to females 112 and 193 in November 2002, to female 356 in September 2004, and to female 357 in October 2004. In all cases, relatively high levels

Figure 9. Fortnightly mean + SEM faecal testosterone concentrations in captive male stoats (*Mustela erminea*; n = 4) exposed to normal photoperiod both during the breeding and non-breeding season.

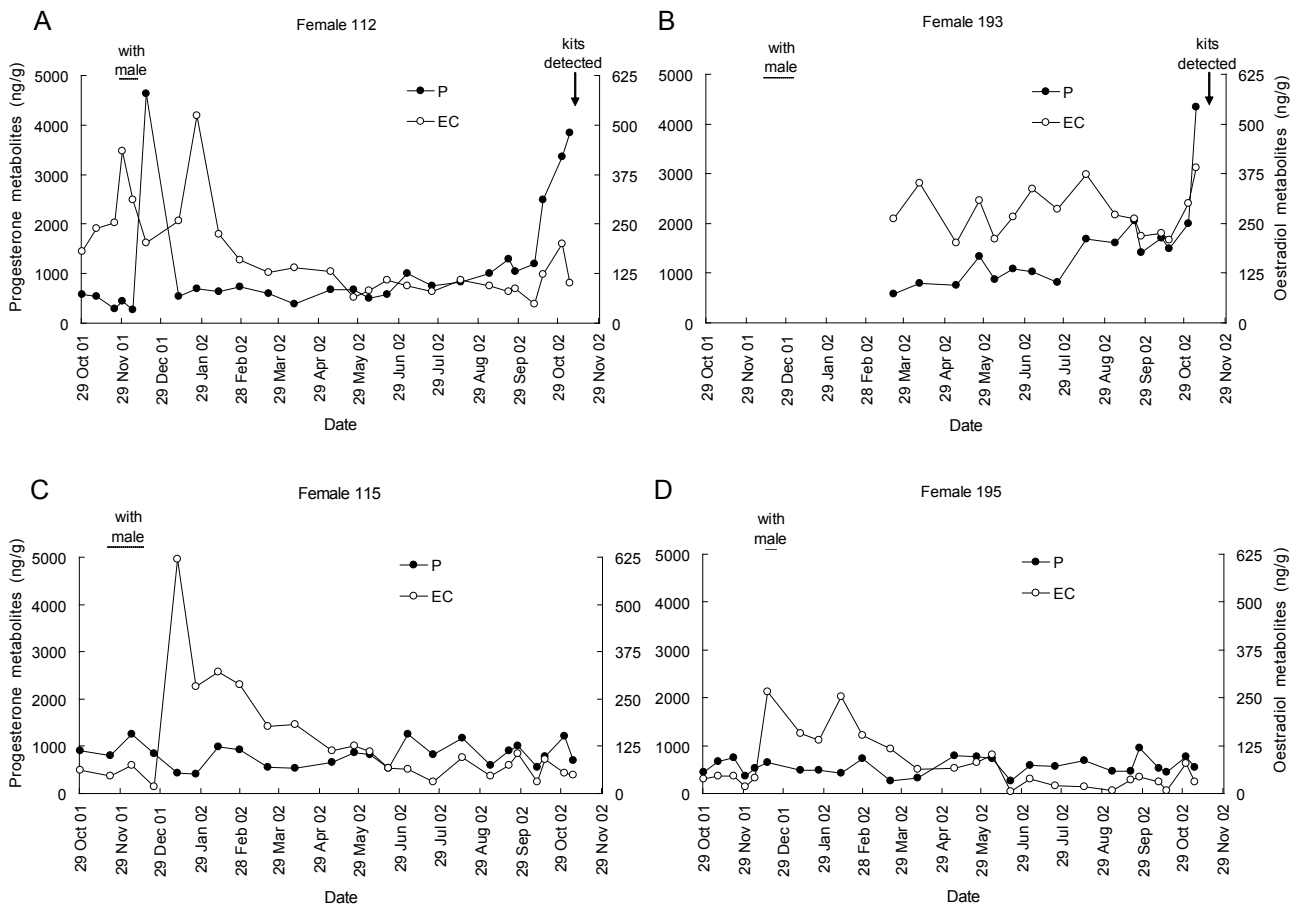
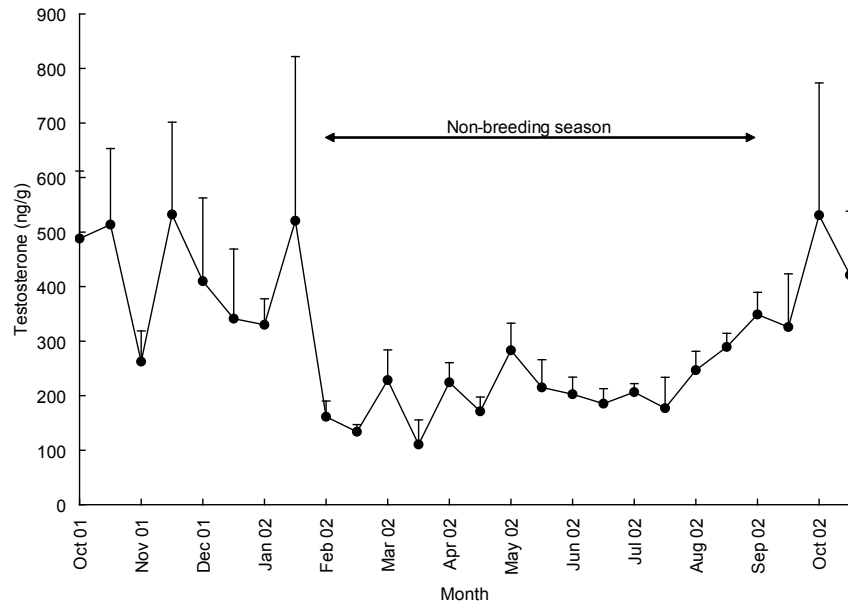


Figure 10. Faecal oestradiol (EC) and progesterone (P) metabolite concentrations of female stoats (*Mustela erminea*): A. 112; B. 193; C. 115; and D. 195. Animals 112 and 193 gave birth during the 2001/02 breeding season; animals 115 and 195 did not. Horizontal lines indicate when females were paired with males for mating. Arrows indicate dates when kits were first detected.

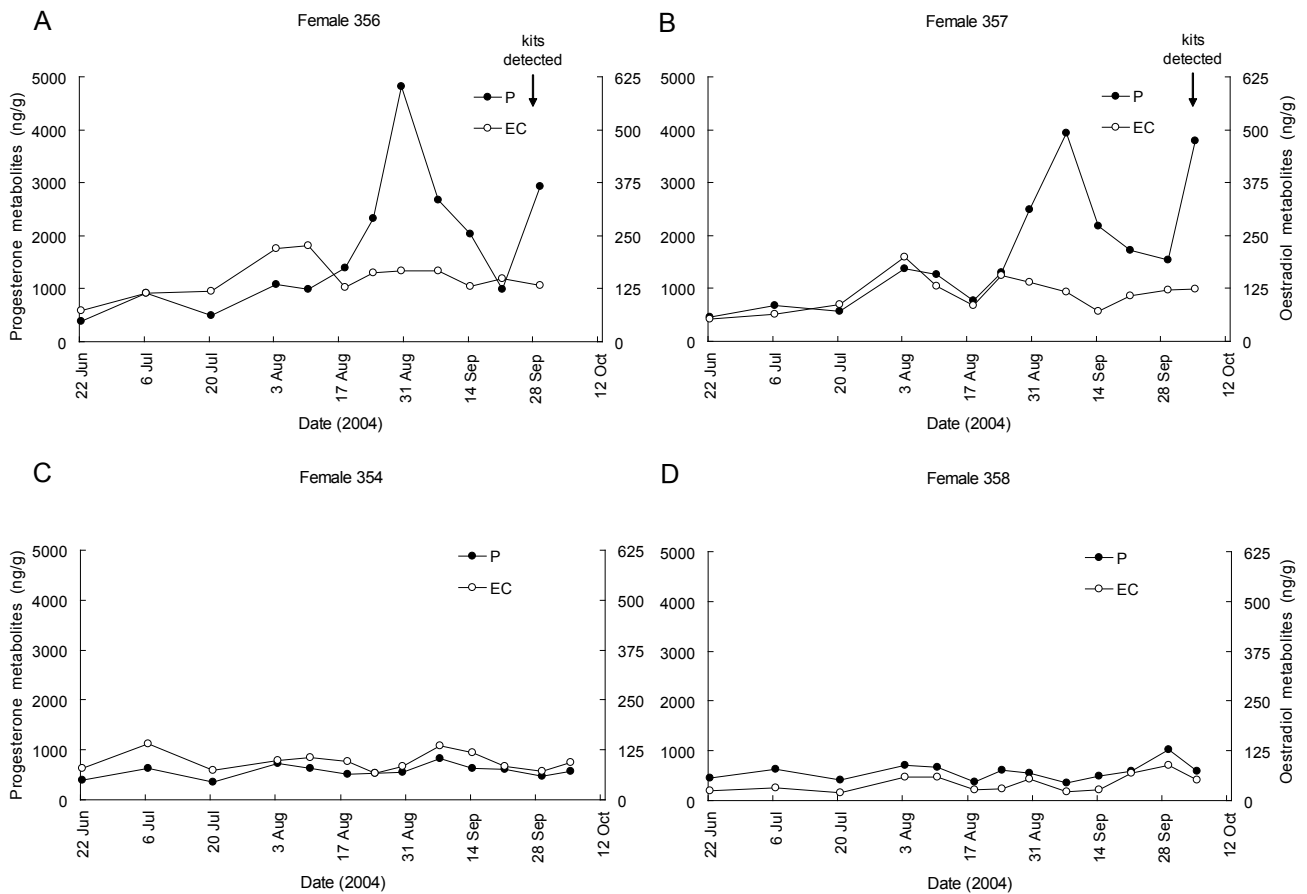


Figure 11. Faecal oestradiol (EC) and progesterone (P) metabolite concentrations of female stoats (*Mustela erminea*): A. 356; B. 357; C. 354; and D. 358. Animals 356 and 357 gave birth during the 2003/04 breeding season; animals 354 and 358 did not. Arrows indicate dates when kits were first detected.

of faecal progesterone metabolites (>2000ng/g) were detected during the month preceding birth (Figs 10A & B and 11A & B).

Faecal hormone monitoring did not, however, help to distinguish between pregnant and non-pregnant stoats before implantation and during the period of embryonic diapause. For female 112, the only female from which a full complement of faecal samples was available from mating through to birth, diapause appeared to correspond to the 9-month period from January to September 2002 between progesterone metabolite peaks (Fig. 10A). However, taking the period between February and July as representative of the period of diapause, mean (\pm SEM) concentrations of faecal oestradiol and progesterone metabolites were higher for the four females that did give birth (142.1 ± 46.4 ng/g and 680.3 ± 77.3 ng/g, respectively) than for the four that failed to give birth (90.2 ± 25.3 ng/g and 575.1 ± 75.2 ng/g, respectively), but the differences were not significant (unpaired sample *t*-tests, $t=0.98$ and 0.98 , $df=6$, $P=0.364$ and 0.367 , respectively).

4.3 PHOTOPERIOD TREATMENT TO ADVANCE SEASONAL REPRODUCTION

Following exposure of stoat males to increased artificial lighting from May 2003, mean testis length (Fig. 12A) and width (Fig. 12B) were consistently greater through to early October than in controls exposed to normal photoperiod. This difference was significant in August (repeated measures ANOVA, $P < 0.05$). A similar trend was observed for mean faecal testosterone levels, with concentrations being significantly higher in photoperiod-advanced than control males in late July and early September (repeated measures ANOVA, $P < 0.05$; Fig. 12C).

The mean percentage of keratinised cells in vaginal washes was higher in photoperiod-advanced than control female stoats, and the difference was significant in August and October (repeated measures ANOVA, $P < 0.05$; Fig. 13A). Although RIA measures of oestradiol from matching faecal samples were generally higher in photoperiod-advanced than control females over the same period, the mean differences were not significant (Fig. 13B).

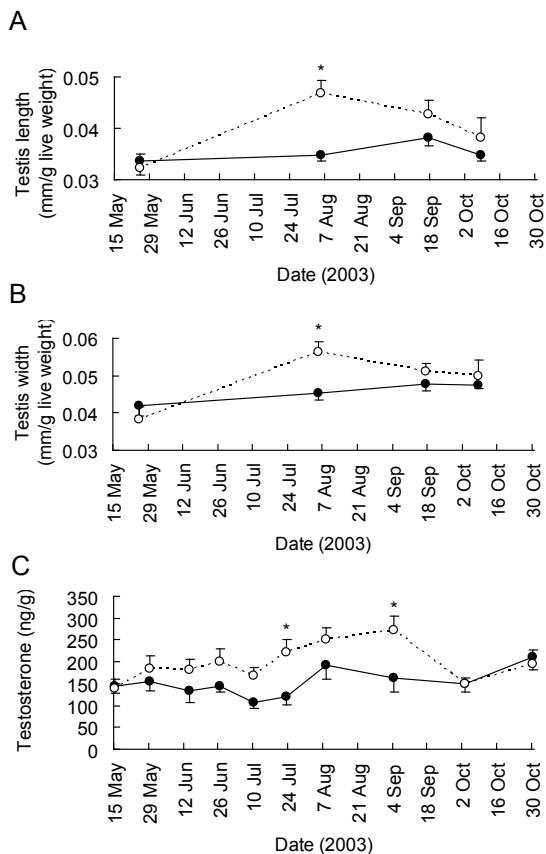


Figure 12. Mean \pm SEM changes in A. testis length, B. testis width, and C. faecal testosterone concentrations for control and photoperiod-advanced male stoats (*Mustela erminea*; $n = 6$ per group). Asterisks indicate significant differences ($P < 0.05$).

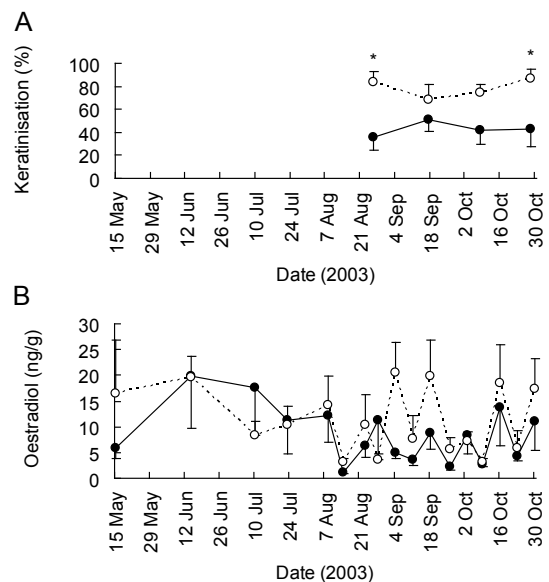


Figure 13. Mean \pm SEM changes in A. percentage of keratinised cells, and B. faecal oestradiol concentrations for control and photoperiod-advanced female stoats (*Mustela erminea*; $n = 6$ per group). Asterisks indicate significant differences ($P < 0.05$).

5. Discussion

Faecal hormone assays have been established to non-invasively monitor the major hormones of reproduction in captive stoats (*Mustela erminea*). The extraction protocol developed proved to be an effective and reliable method for extracting testosterone, oestradiol and progesterone metabolites from stoat faecal samples based on the achievement of extraction efficiencies of around 70% or greater for all three assays. The findings from the HPLC indicate that for the stoat, testosterone remains in its native form whereas oestradiol and progesterone are metabolised before excretion in faeces. In contrast, HPLC and immunoactivity analyses of black-footed ferret faecal samples indicated that oestradiol was excreted in its native form, whereas progesterone and testosterone apparently underwent considerable metabolism (Brown 1997). This supports earlier findings that there is considerable variation in steroid metabolite concentrations excreted in faeces even among closely related species (Schwarzenberger et al. 1996). Our results indicate that the testosterone EIA is suitable for monitoring testosterone in male stoat faeces, and that our oestrone conjugate and pregnane EIAs are appropriate for monitoring oestradiol and progesterone metabolite concentrations, respectively, in female stoat faeces.

Physiologically induced changes in reproductive hormone activity were measured in faecal extracts, and so the EIAs developed in this study were biologically validated for stoats. The time-lag between hormone challenge and peak faecal steroid responses measured by EIA has been examined for several carnivore species. The latency period to peak faecal androgen varied from 1 to 3 days after luteinising-hormone-releasing hormone challenge of spotted hyenas (Dloniak et al. 2004). Faecal glucocorticoid metabolites increased sharply to reach peak concentration 1–2 days after adrenocorticotrophic hormone challenge of various carnivore species (Himalayan black bear, domestic cat, cheetah, clouded leopard, black-footed ferret, slender-tailed meerkat and red wolf; Young et al. 2004). In our study, peak testosterone, oestradiol and progesterone metabolites were measured in stoat faecal extracts 24 h after hormone challenge.

This study represents the first circannual evaluation of testicular and ovarian steroidogenic activity, conducted entirely non-invasively, in the stoat. The temporal testosterone, oestradiol and progesterone metabolite excretion patterns were found to correspond with seasonally mediated reproductive events known for stoats (reviewed by Amstislavsky & Ternovskaya 2000) and observed in our own colony (O'Connor et al. 2006). This further validates the EIAs developed in this study for monitoring stoat male and female reproductive function.

Like black-footed ferrets (Brown 1997), the longitudinal profiles of male stoats revealed a seasonal pattern. Increases in testosterone production began several months before the initiation of oestrus in females, presumably to activate spermatogenic activity. Faecal testosterone levels increased between August and October, and a similar trend in serum testosterone concentration was reported over the 3 months leading up to the breeding season of stoats in the Northern Hemisphere (Gulamhusein & Tam 1974). The observed increase

in faecal testosterone in this study corresponds with previous measures of seasonal testicular activity in stoats, where the size and weight of the testes, and the quality and quantity of epididymal sperm increase as the breeding season approaches (King & Moody 1982; O'Connor et al. 2006).

In females, the changes in oestradiol and progesterone metabolite secretion around oestrus detection and after mating were similar to those reported for the black-footed ferret and other carnivores (Young et al. 2001). The high levels of faecal progesterone metabolites during the month before birth probably correspond with corpus luteum reactivation and post-implantation pregnancy in the stoat. The highest levels of plasma progesterone were also related to implantation in another mustelid with delayed implantation, the American mink (Stoufflet et al. 1989). Our results indicate that pregnancy can now be confirmed in stoats before the birth of kits by monitoring for increases in faecal progesterone metabolites that coincide with implantation.

However, pregnancy could not be unequivocally detected in stoats during embryonic diapause from measures of faecal oestradiol and progesterone metabolites. This supports earlier findings that plasma progesterone levels were not different between pregnant and non-pregnant female stoats during diapause (Gulamhusein & Thawley 1974). Faecal progesterone metabolite levels were also similar between pregnant and pseudopregnant ferrets (Brown 1997), but more frequent sampling of a larger number of animals in a later study did reveal a difference (Young et al. 2001). The same might be true for stoats if a similar approach was undertaken in the future.

The photoperiod study demonstrated the value of faecal hormone assays to non-invasively monitor the manipulation of stoat reproduction. Increases in faecal testosterone corresponded to increases in testis size, providing additional information about the effects of advancing photoperiod on male reproductive seasonality in stoats, as reported in other species (e.g. Pallus' cats; Brown et al. 2002). It was not surprising that the testosterone RIA was also suitable for measuring faecal testosterone in lieu of the EIA method, given that testosterone is the main excretory product detected in stoat faeces. However, the EIA assay would circumvent the need to use radioactive reagents in future studies on stoats to monitor testicular function.

Measures of the percentage of keratinised cells were useful for detecting the early onset of oestrus in photoperiod-advanced females. This extends the findings of O'Connor et al. (2006), where 5/6 of these same photoperiod-advanced females were detected to be in oestrus between August and October from observations of vulval swelling, compared with 0/6 controls. However, measures of faecal oestradiol could not distinguish between these groups. This could be due to several factors, including small animal sample size and the infrequency of faecal sampling and oestrus detection (McLane 2004). Faecal samples were collected on the same day as other assessments were made, and in black-footed ferrets this has been shown to be less reliable than 4-day mean values of faecal oestradiol concentrations for correlation with changes in vulval size and vaginal cytology as indicators of oestrus (Young et al. 2001). Moreover, given that HPLC results revealed that only a relatively small amount of native oestradiol is excreted in stoat faeces, it would be more appropriate to use the oestrone conjugate EIA rather than the oestradiol RIA to examine the oestrous period of stoats in the future.

6. Conclusions

- A reliable procedure has been established for extracting testosterone, oestradiol and progesterone metabolites from stoat faecal samples. HPLC analysis of extracts revealed that testosterone remains in its native form, whereas oestradiol and progesterone are metabolised before excretion in stoat faeces.
- A testosterone EIA was validated for monitoring stoat male (testicular) function. Faecal testosterone excretion was found to be seasonal, with levels higher during the breeding season (September–January) than outside it (February–August).
- An oestrone conjugate and a progesterone EIA were validated for monitoring stoat female (ovarian) function. Increases in faecal oestradiol and progesterone metabolites were found to be coincident with oestrus and implantation, respectively. However, with the limited samples available, neither assay could detect differences between pregnant and non-pregnant females during embryonic diapause and before implantation.
- Measures of faecal testosterone, but not of faecal oestradiol, by RIA supported the assessments of testis size and showed that photoperiod treatment had advanced seasonal reproduction in captive stoats.

7. Recommendations

Based on the results reported in this study, the authors make the following recommendations:

- Stoat researchers could adopt these non-invasive hormone monitoring techniques to improve captive breeding management of animals and to evaluate the effects of manipulation or treatment with fertility control agents on stoat reproductive function.
- More frequent faecal sampling from a larger sample size of female stoats is needed to establish a more comprehensive understanding of how measures of faecal oestradiol and progesterone metabolites relate to key reproductive events, especially from oestrus through to the maintenance of pregnancy during the period of embryonic diapause.
- Further research could establish a non-invasive method for monitoring stress in stoats, to support captive breeding programmes and to evaluate the humaneness of current and emerging stoat control methods on animal welfare.

8. Acknowledgements

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How can we monitor reproductive hormones in stoats?

A technique was developed to non-invasively monitor reproductive cycles in captive stoats (Mustela erminea). Reproductive hormone metabolites were successfully extracted and measured from stoat faecal samples. It was found that faecal testosterone levels were highest in males during the breeding season and increases in faecal oestradiol metabolites occurred when females were 'in heat' (oestrus). The concentration of faecal progesterone metabolites was also used to confirm pregnancy in stoats following implantation and before the birth of kits. Non-invasive reproductive hormone monitoring will be a valuable tool to improve captive breeding effort and to evaluate the effects of manipulation or fertility controls on the reproductive function of stoats in the future.

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