Stoat artificial reproductive technologies

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

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ABSTRACT

Artificial reproductive technologies in stoats (*Mustela erminea*) are currently being developed in New Zealand to control and manipulate stoat reproduction in captivity. In this study, methods were established for liquid (short-term) and frozen (long-term) storage of stoat sperm. Frozen-thawed sperm were shown to be biologically competent *in vitro*, based on the maintenance of sperm motility and acrosome integrity after culture. A non-surgical artificial insemination technique is in development to confirm that frozen-thawed stoat sperm are capable of fertilising eggs *in vivo*, but its success is reliant on developing methods of inducing ovulation in females. Ovulated eggs and embryos were only recovered after mating oestrous females. Treatment of anoestrous females with pregnant mare serum gonadotrophin (PMSG) did not reliably stimulate ovarian follicle development and failed to induce ovulation. Treatment of oestrous females with PMSG did not improve the reliability and yield of eggs and embryos recovered after natural mating, or their capacity to develop *in vitro* compared with untreated controls. For the first time, *in vitro* culture and the development of *in vivo* derived one- to four-cell embryos to the blastocyst stage was achieved in stoats, with up to 8% of these hatching. This culture system did not support the viability of diapausing blastocysts *in vitro*, suggesting that alternative substrates, nutrients and/or co-culture with uterine cell layers may be required. The findings from this study will enhance our ability to breed stoats in captivity and will help establish robust protocols for efficient testing and prioritisation of reproduction-based targets for stoat fertility control.

Keywords: stoat, *Mustela erminea*, reproductive technology, sperm preservation, hormone treatment, artificial insemination, *in vitro* culture

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1. Introduction

Stoats (*Mustela erminea*) are one of New Zealand's most destructive predators, responsible for the decline of several native iconic bird species (reviewed by King & Powell 2007). Current control techniques, such as labour-intensive trapping and ground-based non-specific poisoning, are failing to deal adequately with the problem, and raise serious concerns about non-target impacts, humaneness and environmental risk (Parkes & Murphy 2004). Fertility control is one approach being developed as a sustainable and potentially species-specific method of stoat control for use over large spatial scales with enhanced acceptability and safety (Norbury 2000). Recent modelling has also indicated that an integrated approach, combining fertility control with conventional methods, may offer a cost-effective solution for managing stoat overabundance in the long term (Barlow & Choquenot 2002).

Stoats have an unusual breeding system (King & Murphy 2005) that lends itself to disruption at various stages. In New Zealand, female stoats (both adults and 3-week-old young) come into 'heat' (or oestrus) and mate during spring (September–November). Mating induces the release (or ovulation) of mature eggs 3–4 days later, and if fertilisation is successful, embryos undergo cleavage and development during passage through the oviduct (Amstislavsky et al. 1993, 1994). Embryos reach the uterus by Day 11 after mating, as fluid-filled balls of c. 250 cells called blastocysts. During the next stage of pregnancy known as diapause, the blastocysts grow but differentiation is arrested, and they float free in the uterus for c. 8–9 months. In August, blastocysts resume differentiation and implant into the uterine horns, and pregnancy continues on to the birth of multiple young c. 4 weeks later. Females rear young alone, which are weaned at 6–8 weeks of age. Stoats can produce only one litter a year, so disruption of stoat reproduction has the potential to dramatically increase control effectiveness.

Several agents that have the potential to disrupt stoat reproduction have already been identified (Hinds et al. 2000). Crucial to testing their effects on fertility is having reproductively functioning stoats in captivity. One approach is to establish a captive-stoat breeding colony. Mating, full-term pregnancy and birth of stoats in captivity was recently achieved for the first time in the Southern Hemisphere (O'Connor et al. 2006). To support this work, the development of artificial reproductive technologies to control and manipulate stoat reproduction has been investigated. This same approach is being used for possum (*Trichosurus vulpecula*) biocontrol research, where superovulation and artifical insemination are used routinely as a bioassay for testing fertility control vaccines (Molinia et al. 2007). The focus of this study was to develop methods for sourcing stoat gametes (sperm and eggs) and embryos. This research aimed to significantly broaden our knowledge of the key features of stoat reproduction and enhance our ability to breed stoats in captivity, as steps towards the establishment of robust protocols for efficient testing and prioritisation of reproduction-based targets for stoat fertility control.

2. Objectives

The aim of this research was to develop artificial reproductive technologies in stoats that would provide a platform for future research on fertility control and supplement captive breeding of stoats for research by:

- Developing an optimum technique for the preservation of stoat sperm and studying the biological competence of frozen-thawed sperm.
- Performing hormone treatments, mating-induced ovulation and/or artificial insemination of stoats to produce eggs and embryos both during and outside the breeding season.
- Determining *in vitro* culture media and conditions required for maintaining the viability of stoat embryos and blastocysts.

3. Methods

Wild-caught stoats were housed in cages (60 cm × 150 cm × 90 cm high) under an outside shelter at the Animal Facility at Landcare Research, Lincoln. Each stoat was provided with a nest box (40 cm × 33 cm × 15 cm high) containing shredded paper 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 supplied with water *ad libitum*.

All research involving stoats was performed in accordance with the 1999 Animal Welfare Act of New Zealand and approved by the Animal Ethics Committee of Landcare Research, Lincoln (Approval Numbers 01/08/02 and 03/11/02).

3.1 SPERM PRESERVATION

3.1.1 Liquid and frozen sperm storage

Between September and January, eight sexually mature male stoats were used to provide sperm for the development of a technique for liquid and frozen sperm storage (Fig. 1). The cauda epididymides were removed from euthanased animals, macerated with a scalpel blade, then overlayed with Krebs-Ringer-Phosphate (KRP) media. Motile sperm were allowed to 'swim-up' into this medium at 37°C in 5% CO_2 in air for 1 h; the medium was then centrifuged (300*g* for 10 min at room temperature) before resuspending sperm in fresh KRP medium to a concentration of 20×10^6 sperm/mL.

To examine whether time of exposure to glycerol during processing affected sperm preservation, stoat sperm were diluted in one step (constant exposure to glycerol) or two steps (exposure to glycerol after cooling)

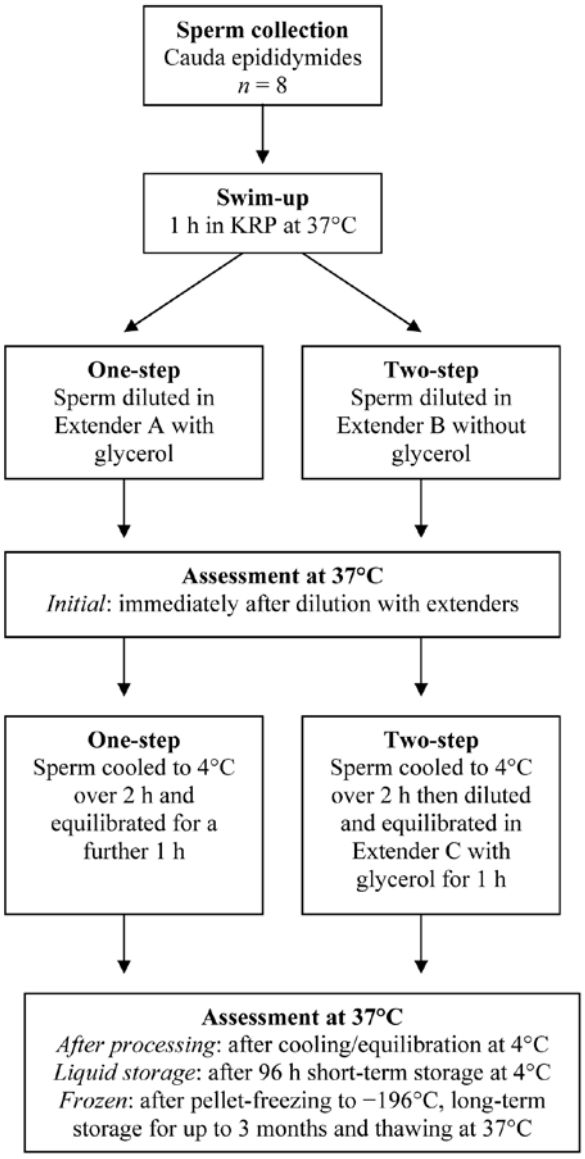

Figure 1. Protocol used for the development of liquid and frozen storage of stoat (*Mustela erminea*) sperm using a one- and two-step dilution method.

using modified Tris-fructose-citric acid-egg yolk extenders, which have been used for successfully freezing cat (*Felis catus*) semen (Tsutsui et al. 2000; Table 1). For the one-step method, sperm were diluted with an equal volume of extender A before cooling to 4°C over 2 h; they were then incubated for a further 1 h at 4°C to allow equilibration. For the two-step method, sperm were diluted with a half-volume of extender B before cooling to 4°C over 2 h. Sperm were then diluted with an equivalent half-volume of extender C and allowed to equilibrate for a further 1 h at 4°C. This meant that, after processing, the final concentrations of sperm (10×10^6/mL), glycerol (3.5%) and egg yolk (10%) were identical after one- or two-step dilution.

Processed sperm diluted by the one- and two-step methods were each split into two sub-samples. One sample was stored in liquid form at 4°C for 96 h. The other sample was frozen into 0.25-mL pellets on dry ice for 15 min, transferred into liquid nitrogen and frozen stored at –196°C for up to 3 months. For use, pellets were transferred to dry glass test tubes and thawed by vigorous shaking in a waterbath at 37°C for 30 s.

To examine the effect of one- and two-step dilution, aliquots of stoat sperm were rewarmed to 37°C and assessed for percentage motility (0–100%), vigour scored on a scale of 0 (no movement) to 5 (fast progressive movement), and percentage of intact acrosomes (0–100%) using phase contrast microscopy. Assessments were undertaken immediately after dilution with extenders, after processing, after liquid storage, and after thawing of samples held in frozen storage.

3.1.2 Biological competence of frozen-thawed sperm

Fertilisation and the birth of live young following artificial insemination is the 'gold standard' for confirming the biological competence of frozen-thawed sperm. However, there is no report of artificial insemination being attempted in stoats (until the present study) and in lieu of a reliable protocol being developed, a simple alternative is to monitor key aspects of sperm function, such as motility and acrosome integrity, over time *in vitro*.

During September–January, three sexually mature male stoats were used to provide sperm to assess the biological competence of frozen-thawed stoat sperm. Sperm were diluted by the one- and two-step methods and frozen-stored in liquid nitrogen for at least 1 week prior to thawing, as detailed above. Frozen-thawed sperm from each male were incubated at 37°C for 2 h, and aliquots (in triplicate) were subsampled at 0, 30, 60 and 120 min to assess sperm motility and acrosome integrity, as outlined above.

TABLE 1. COMPOSITION OF EXTENDERS USED FOR ONE- AND TWO-STEP DILUTION OF STOAT (*Mustela erminea*) SPERM.

INGREDIENTS	ONE-STEP DILUTION	TWO-STEP DILUTION	
	EXTENDER A	EXTENDER B	EXTENDER C
Tris(hydroxymethyl)aminomethane	2.4 g	2.4 g	2.4 g
Fructose	1.0 g	1.0 g	1.0 g
Citric acid	1.3 g	1.3 g	1.3 g
Penicillin potassium	100 000 U	100 000 U	100 000 U
Streptomycin sulphate	0.1 g	0.1 g	0.1 g
Glycerol	7 mL	–	14 mL
Egg yolk	20 mL	20 mL	20 mL
Distilled water (to final volume)	100 mL	100 mL	100 mL

3.2 ARTIFICIAL INSEMINATION

Three female stoats were used during October–December to develop artificial insemination techniques. When evidence of oestrus was obtained by vaginal smears, mechanical simulation of mating (up to six intromission events over 10 min) was undertaken on anaesthetised females using a glass rod, to induce ovulation, based on an approach that was successful in ferrets (Bibeau et al. 1991). The glass rod used was approximately the same diameter and length as the stoat penis. While females were under anaesthesia, frozen-thawed sperm were inseminated. Frozen sperm were thawed at 37°C for 30 s and the sperm motility was checked by phase contrast microscopy. Insemination was performed using a 2-cm-long cannula (1.2 mm in diameter) attached to a syringe containing at least 4×10^6 motile sperm in a 0.5-mL dose. The inseminating pipette was inserted as deeply as possible into the central region of the cervix without using force and females were kept inclined head downwards for a few minutes to avoid semen reflux. Mechanical simulation of mating and insemination were repeated 24 h later to improve the chances of inducing ovulation and achieving fertilisation. Over the next 2 consecutive weeks, females were checked again for oestrus by vaginal smears. If females were still in oestrus, induced ovulation treatment and insemination was repeated 0 h and 24 h later (as above). Animals were euthanased 1 week after the last inseminations to determine if the technique was successful.

3.3 PRODUCTION OF EGGS AND EMBRYOS

In a series of pilot studies that were undertaken to examine whether hormone treatment of female stoats outside the breeding season could be used to reliably produce eggs and embryos, six non-pregnant female stoats were treated with 1.5, 3.0 or 6.0 IU pregnant mares serum gonadotrophin (PMSG) during April 2002 (A. Glazier, Landcare Research, unpubl. data). Post-mortem analysis of ovaries revealed that these treatments failed to induce ovulation in any female. Treatment with 3.0 IU PMSG stimulated recruitment and development of ovarian follicles in two females, but only

seven oocytes were collected from large follicles (> 0.5 mm) post-mortem, of which three were classified as mature based on extrusion of the first polar body. Instead, it was shown that the most efficient method for obtaining embryos was to mate stoats during the breeding season; blastocysts were also successfully obtained outside the breeding season by mating stoats previously exposed to a long-day photoperiod that simulated summer day length (O'Connor et al. 2006). Accordingly, we examined whether PMSG treatment of oestrous females could improve the reliability and/or yield of eggs and embryos obtained after natural mating.

3.3.1 Hormone treatment of oestrous females

Eighteen oestrous females were used to investigate the effect of hormone treatment on egg and embryo yields after natural mating. From September to December 2003, females were examined twice weekly to confirm oestrus, as indicated by swelling of the vulva and a high ratio of keratinised to non-keratinised cells in vaginal smears (O'Connor et al. 2006). Oestrous females were randomly allocated into two groups of nine females. One group received an intramuscular injection of 10 IU PMSG; the other (control) group did not receive any hormone treatment. Each female was paired with a single male for up to 2 consecutive days to allow animals to mate. Mating was confirmed from vaginal smears of females that indicated they were no longer in oestrus. Females were euthanased 5–6 days after mating, and eggs were flushed from the oviducts and uterus with Dulbecco's phosphate buffered saline (DPBS) supplemented with 1 g/L glucose and 0.8% bovine serum albumin (BSA). Eggs were incubated *in vitro*, as described below (section 3.4.1), to examine the effect of PMSG pre-treatment of females on egg development in culture. The batches of PMSG used in this study were the same as those that successfully induced recruitment and development of ovarian follicles in brushtail possums (Molinia et al. 2007).

3.4 *IN VITRO* CULTURE

3.4.1 Embryos

The eggs recovered from the PMSG-treated ($n = 9$) and control ($n = 9$) oestrous females that were mated as detailed above (section 3.2.1) were used to compare two media for culturing stoat embryos. All eggs recovered were evaluated at 64× magnification using an unheated stage mounted on a stereomicroscope. The developmental stage of eggs was recorded and eggs were photographed. Eggs classified as one- to four-cell embryos were divided into two groups of four to six embryos and put into separate wells of the same four-well culture plate. The groups of embryos were cultured simultaneously in one of the following two media:

- TCM-199 supplemented with 10% foetal bovine serum, 0.25 mM pyruvate and 40 µg/mL gentamycin
- Synthetic Oviduct Fluid supplemented with 0.8% BSA

The embryos were placed in droplets of medium, covered with embryo culture grade mineral oil and incubated at 38.5°C under 5% CO_2 in 95%

air. An incubation temperature of 38.5°C was chosen in accordance with the normal body temperature of stoats (S. Amstislavsky, Russian Academy of Sciences, pers. comm. August 2003). The medium was replaced every 48 h to maintain optimal culture conditions. Some embryos were removed at different stages during culture and stained with 10 µg/mL bisbenzimide (Hoechst 33342) for 5 min, washed with PBS and assessed for stage of development using fluorescence microscopy. The capacity of embryos to develop *in vitro* was compared between the two culture media.

3.4.2 Diapausing blastocysts

Four pregnant female stoats were used for *in vitro* culture of diapausing blastocysts. Females were euthanased during the period of embryonic diapause and expanded blastocysts were recovered from the uteri and placed in culture. The same two media and culture protocols were used as for embryo culture (section 3.4.1), and the capacity of the diapausing blastocysts to develop *in vitro* was examined.

4. Results

4.1 SPERM PRESERVATION

4.1.1 Liquid and frozen sperm storage

There were no differences in sperm motility or vigour between the one- and two-step dilution methods immediately after dilution of stoat epididymal sperm with extenders, after processing, after liquid storage or after thawing of samples held in frozen storage (Wilcoxon signed ranks tests: $T^+ = 3–27$, all $P > 0.05$; Table 2). Similarly, there was no difference in the proportion of sperm with intact acrosomes at any of the stages, except after frozen storage, where there was a significantly higher proportion of intact acrosomes for the two-step than the one-step method (Table 2). However, this small difference was not sustained after incubation of frozen-thawed sperm over 2 h (see below).

TABLE 2. EFFECT OF ONE- AND TWO-STEP DILUTION ON THE MOTILITY, VIGOUR AND ACROSOME INTEGRITY OF STOAT (*Mustela erminea*) SPERM.

Values are means ± SEM. The two values assigned different letters are significantly different from each other (Wilcoxon signed ranks tests: $T^+ = 34$, $P = 0.0234$).

	MOTILITY (%)		VIGOUR (0–5)		INTACT ACROSOMES (%)	
	ONE-STEP	TWO-STEP	ONE-STEP	TWO-STEP	ONE-STEP	TWO-STEP
Initial	71.3±0.8	75.0±1.3	3.5±0.2	2.9±0.1	96.5±0.7	95.1±0.6
After processing	56.3±0.8	59.4±1.1	3.0±0.2	3.6±0.3	80.3±0.4	80.9±0.4
Liquid storage	55.6±2.2	58.8±0.8	2.9±0.2	3.6±0.2	60.9±1.7	60.1±0.6
Frozen storage	40.6±2.2	38.1±2.5	2.6±0.2	2.8±0.2	76.4±1.2a	81.4±0.8b

4.1.2 Biological competence of frozen-thawed sperm

Irrespective of how samples were diluted prior to cryopreservation, there were decreases in motility and acrosome integrity of frozen-thawed stoat sperm over the 2-h incubation period, but their magnitude was small. For the one-step method, there was a 5.6% drop in motility (linear mixed effects model: $t = 5.03$, df = 83, $P < 0.001$) and a 3.7% drop in acrosome integrity (linear mixed effects model: $t = 4.47$, df = 41, $P < 0.001$). For the two-step method, post-thaw motility declined by 3.1% (linear mixed effects model: $t = 3.88$, df = 83, $P < 0.001$) and acrosome integrity by 7.3% (linear mixed effects model: $t = 4.19$, df = 41, $P < 0.001$). However, there was no difference in the rate of those declines between the one-and two-step dilution methods (motility: $t = 1.76$, df = 166, $P = 0.081$; acrosome integrity: $t = 1.85$, df = 82, $P = 0.067$).

4.2 ARTIFICIAL INSEMINATION

Mechanical simulation of mating and insemination were undertaken over 3 consecutive weeks; however, all three females remained in oestrus and no eggs were recovered from them post-mortem. Each insemination lasted 10–20 min to allow stimulation of the cervix, placement of the cannula and semen deposition. The technique was not compromised by semen quality *per se*, since the proportion of motile frozen-thawed sperm was high at the time of insemination (57.0 ± 4.4%) and motile sperm were still recovered from the reproductive tract up to 3 days later in vaginal smears.

4.3 PRODUCTION OF EGGS, EMBRYOS AND BLASTOCYSTS

4.3.1 Hormone treatment of oestrous females

Vaginal smears of all 18 females paired with males revealed that they were no longer in oestrus, suggesting that mating was probably successful. Recent ovulation sites were detected in ovaries, and eggs were recovered from the oviducts of eight (of nine) PMSG-treated and eight (of nine) control females harvested 5–6 days after mating. No eggs were recovered from two females, either because they were lost during post-mortem processing or because the females failed to mate properly and ovulation was not successfully induced. There was no significant difference in the mean number of eggs recovered ($F = 0.17$, df = 1,16, $P = 0.683$) or eggs classified as unfertilised or degenerate ($F = 2.37$, df = 1,16, $P = 0.143$) between PMSG-treated and control females after mating (Table 3). Eggs classified as normal one- to four-cell embryos continued development during *in vitro* culture and there was no significant difference in the proportion of embryos surviving through to cleavage ($F = 0.26$, df = 1,14, $P = 0.616$), morulae ($F = 1.38$, df = 1,14, $P = 0.260$) and then blastocyst stages ($F = 2.16$, df = 1,14, $P = 0.164$) between PMSG-treated and control females (Fig. 2).

TABLE 3. EGGS RECOVERED FROM THE OVIDUCTS OF PMSG-TREATED AND CONTROL FEMALE STOATS (*Mustela erminea*) AFTER MATING.

	PMSG	CONTROL
Number of females	9	9
Total eggs	50	44
Mean (SEM lower limit–upper limit)	5.6 (4.4–6.8)	4.9 (3.8–6.0)
Range	0–12	0–8
Total unfertilised or degenerate eggs	5	1
Mean (SEM lower limit–upper limit)	0.6 (0.3–0.9)	0.1 (0–0.3)
Range	0–3	0–1

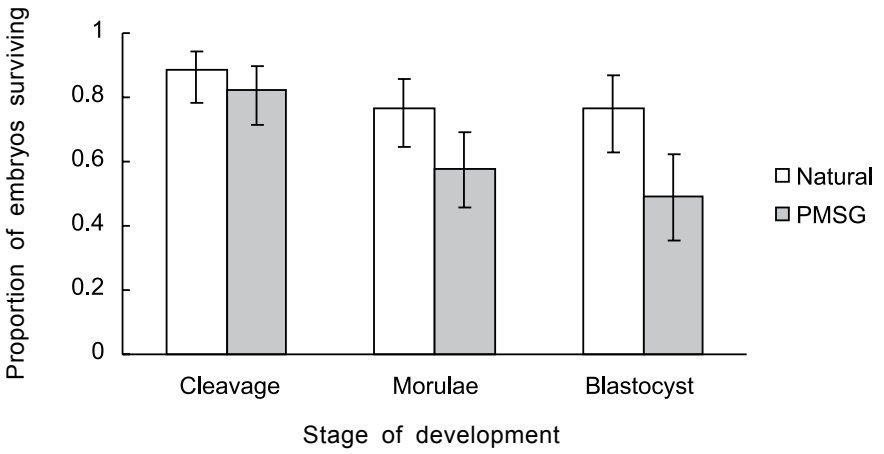

Figure 2. Effect of PMSG treatment and no hormone treatment (Natural) of female stoats (*Mustela erminea*) prior to mating on the capacity of one- to four-cell embryos to develop during *in vitro* culture. Data are pooled for TCM-199 and SOF media used for culture, and are presented as the mean proportion of embryos surviving from the start of culture ± SEM.

4.4 *IN VITRO* CULTURE

4.4.1 Embryos

In total, 94 eggs were recovered from the oviducts of PMSG-treated and control females 5–6 days after mating (Table 3). More than 90% (88/94) of these were classified as normal one- to four-cell embryos, and there was no interaction between hormone treatment and medium type on the developmental capacity of embryos *in vitro*. There was no difference in the proportion of embryos surviving through to cleavage ($F = 0.23$, df = 1,14, $P = 0.640$), morulae ($F = 0.18$, df = 1,14, $P = 0.680$) and then blastocyst stages ($F = 0.72$, df = 1,14, $P = 0.409$) between TCM-199 and SOF media used for culture (Fig. 3). The development status of embryos viewed under phase contrast microscopy was confirmed following the fluorescent staining of nuclei (Fig. 4). More than 63% of embryos (56/88) developed to the blastocyst stage, including a small proportion (up to 8%) that had hatched after 120 h of *in vitro* culture.

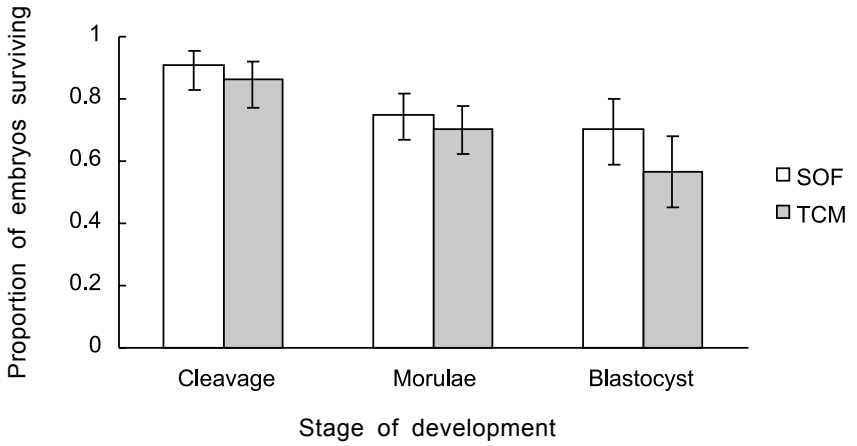

Figure 3. Effect of TCM-199 and SOF media on the capacity of one- to four-cell stoat (*Mustela erminea*) embryos recovered from mated females to develop during *in vitro* culture. Data are pooled for embryos recovered from PMSG and untreated females prior to mating, and are presented as the mean proportion of embryos surviving from the start of culture ± SEM.

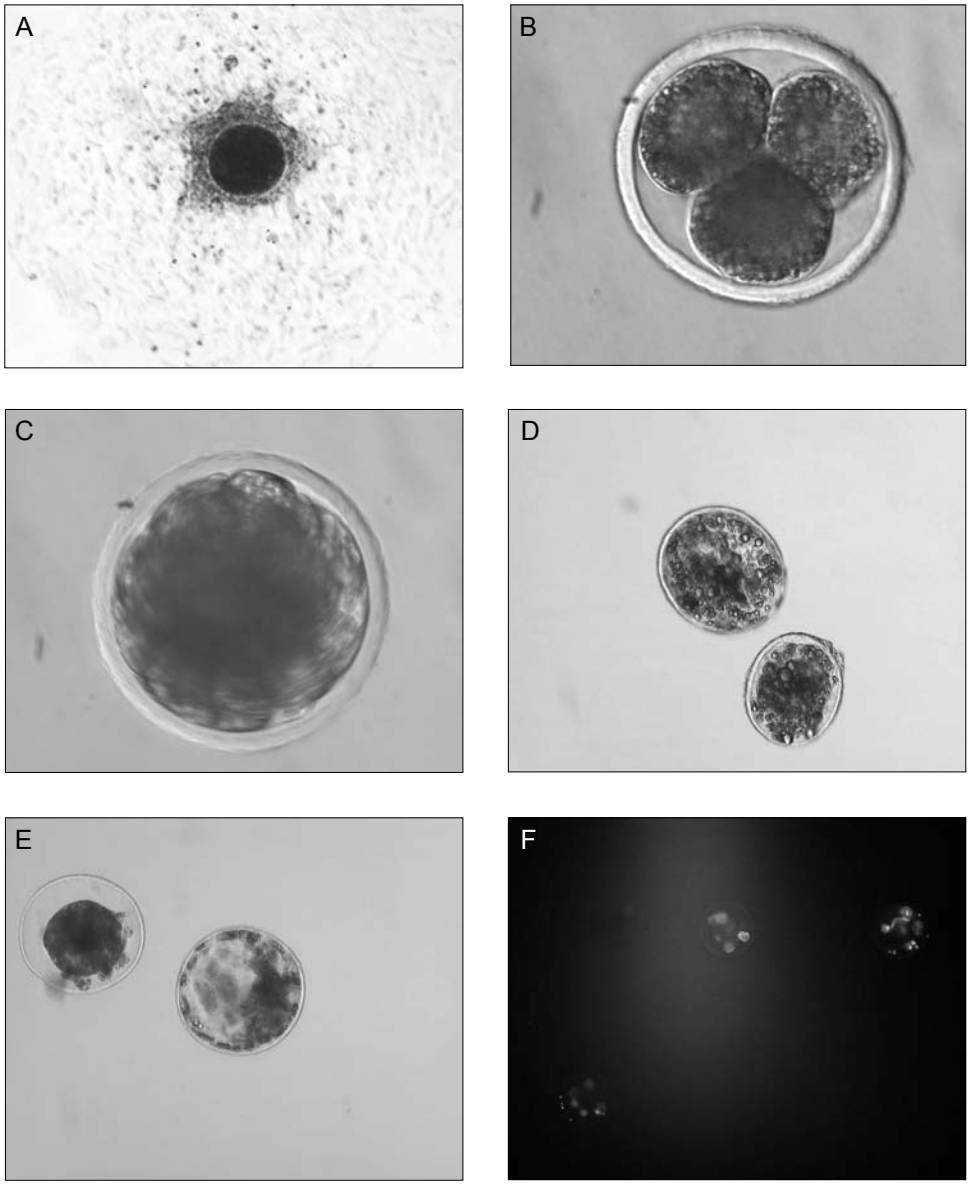

Figure 4. Stoat (*Mustela erminea*) eggs and embryos at various stages of development during *in vitro* culture: A. ovulated egg; B. two- to three-cell embryo; C. morula; D. early blastocyst; E. blastocyst; and F. Hoechst 33342 staining of embryos (blue under UV light) at the four- to eight-cell stage of development.

4.4.2 Diapausing blastocysts

To increase the proportion of blastocysts that hatch under *in vitro* culture conditions, the culture of blastocysts recovered from female stoats during embryonic diapause was explored. A total of 35 expanded blastocysts were recovered from four wild-caught stoats brought into captivity, with fewer than 9% (3/35) classed as degenerate. After culture for 2 h in either TCM-199 or SOF media, all cells collapsed into the centre of each blastocyst, and by 24 h these cells had started to degenerate. Thus, although the culture system supported the development of early-stage embryos, it did not support the on-going viability of diapausing blastocysts *in vitro*.

5. Discussion

This is the first published report outlining methods for storing stoat sperm. Both liquid and frozen storage methods were developed for epididymal sperm collected post-mortem, to circumvent the need for maintaining captive male stoats in reproductive condition during the breeding season. The methods described here would also probably be suitable for storing electroejaculated stoat sperm samples, but this is yet to be tested.

From the initial assessments of sperm quality, it is clear that the swim-up procedure selected a high proportion of good quality sperm. The proportion of sperm with intact acrosomes was >95% after swim-up, which is considerably higher than the proportion (c. 80%) reported for the black-footed ferret (*M. nigripes)*, another mustelid (reviewed by Howard et al. 2003). Our results indicate that motile, vigorous and acrosome intact sperm can be obtained after both liquid (4°C short-term) and frozen (–196°C long-term) storage of epididymal stoat sperm samples with a Tris-fructose-citric acid extender containing a final concentration of 10% egg yolk and 3.5% glycerol. Furthermore, there is no advantage in minimising the time of exposure of stoat sperm to glycerol during cooling and processing, so sperm samples can be diluted using a simple one-step approach.

Data for frozen-thawed stoat sperm from the present study are comparable to those from other closely related ferret species, such as the domestic ferret (*M. putorius furo*), Siberian polecat (*M. eversmanni*) and black-footed ferret (*M. nigripes*), which all produced young following artificial insemination (reviewed by Howard et al. 2003). This suggests that stoat epididymal sperm that are frozen, stored and thawed using the methods described here are likely to be capable of fertilising eggs *in vivo*. However, a successful method for artificial insemination of stoats is required before this can be confirmed.

Our attempt at artificial insemination was not successful because mechanical simulation of mating failed to induce ovulation in oestrous female stoats. Some mustelids, for example ferrets, ovulate spontaneously after handling (Chang 1965). Natural mating of stoats has been observed to occur over 15–50 min with between five and eight intromission events resulting in ovulation

72–96 h later (Amstislavsky et al. 1993). Although our insemination technique mimicked mating, it is possible that anaesthesia may have compromised ovulation as reported in another mating-induced ovulator, the domestic cat (Howard et al. 1992). An important outcome from this study is the development of a simple non-surgical inseminating technique for stoats. Future work should focus on the development of methods to induce ovulation in stoats, such as mating with vasectomised males or treatment of females with agents such as human chorionic gonadotrophin, which has been used successfully in ferrets (Li et al. 2001), prior to performing the insemination technique described here. Once established, artificial insemination will not only offer a cheaper and more reliable alternative to captive mating of stoats to achieve pregnancy, but also promises to be an efficient tool to test fertility control agents for their ability to disrupt egg development and fertilisation.

Only a few studies have investigated the effect of hormonal treatment on ovulation induction in mustelids (Wehrenberg et al. 1992; Amstislavsky et al. 1997; Li et al. 2001). Our results differ from those of Amstislavsky et al. (1997), who reported that treating oestrous female stoats with a single dose of PMSG prior to mating resulted in the recovery of significantly more embryos per female (10.3 ± 0.8) than untreated controls (6.4 ± 1.0). Interestingly, the increased embryo yield was due to stimulation of one and not both ovaries. However, in these studies, young captive-born female stoats were used (26–92 days old), whereas in our studies only wild-caught and acclimatised adult females were used. Treating anoestrous mink (*M. vison*) with PMSG before mating significantly improved the proportion of animals breeding (100% v. 44%), the proportion giving birth (80% v. 33%) and the litter size (4.0 v. 2.6 kits per whelping female) compared with untreated controls (Wehrenberg et al. 1992). Conversely, PMSG treatment did not induce ovulation or reliably stimulate recruitment and development of ovarian follicles in anoestrous stoats (A. Glazier, unpubl. data), which is in agreement with findings elsewhere (S. Amstislavsky, Russian Academy of Sciences, pers. comm. December 2004).

In this study, eggs and embryos were only recovered after mating oestrous females. Further studies examining treatment of acclimatised oestrous stoats of different ages with higher doses of PMSG or other hormones, such as follicle stimulating hormone, which has been used to induce breeding of anoestrous ferrets (Mead & Neirinckx 1989), may improve female reproductive output. Although PMSG treatment did not improve the reliability or yield of eggs and embryos from oestrous stoats after natural mating compared with untreated controls, an important outcome of this work was establishing *in vitro* embryo culture as a method to evaluate techniques for enhancing stoat reproduction.

The rate of embryo development reported in the present study is similar to that of *in vitro* culture of embryos from other mustelids (Li et al. 2001; Lindeberg 2004) and identical to the time required for stoat embryos to reach this developmental stage after natural mating *in vivo* (Amstislavsky et al. 1993, 1994). Li et al. (2001) reported that 64.5% of ferret embryos developed to blastocysts when collected from females after mating and *in vitro* culture in TCM-199. However, transfer of these embryos to the uterus resulted in only a low proportion of female ferrets producing live

offspring. The development of embryo transfer techniques in stoats would show what proportion of blastocysts that are produced after *in vitro* culture continue with normal development *in vivo*.

Most of the stoat embryos that developed into blastocysts were apparently not capable of expanding or hatching in culture. Although 68% of early-stage embryos recovered from the European polecat (*M. putorius*) developed to the morula stage after *in vitro* culture, only 15% developed to blastocysts that expanded and hatched (Lindeberg & Jarvinen 2003; Lindeberg 2004). The authors suggested that this was due to the short exposure of embryos to the normal oviduct environment and hardening of the zona pellucida during *in vitro* culture. In other carnivores, such as the cat, the source of media protein used for *in vitro* culture at least partially explained why most morulae failed to develop to blastocysts (Johnson & Everitt 2000). However, in our study, there was no significant difference between media with foetal bovine serum (in TCM-199) and bovine serum albumin (in SOF) for the development of stoat embryos. It is not known whether the development of egg *in vitro* culture and *in vitro* fertilisation (IVF) for stoats would improve the proportion of embryos that develop in culture. However, when IVF was attempted in the ferret, the proportion of *in vitro* produced embryos that developed to blastocysts in culture was still lower than that achievable after culturing *in vivo* derived embryos (Li et al. 2001).

Nevertheless, an *in vitro* culture system that supports the development of *in vivo* derived early-stage embryos to the blastocyst stage has been achieved for the first time in stoats. This system offers a cost-effective alternative to whole-animal trials for testing fertility control agents targeting early embryo development. Further modification of the culture media based on factors in stoat oviduct or uterine secretions may result in a higher proportion of embryos developing to the blastocyst stage, then expanding and hatching.

The culture system failed to support the development of diapausing blastocysts that all degenerated within 24 h. Nearly one-quarter of mink blastocysts survive beyond 48 h in basic culture media, but enhanced survival and development has been achieved following co-culture with uterine cell lines (Moreau et al. 1995). Post-hatching development of bovine embryos has been achieved after culture in agarose gel tunnels to provide three-dimensional structural support for the developing embryo and high glucose (27.7 mM) for energy to permit rapid growth of the embryo (Brandao et al. 2004). The development of a specialised culture medium based on the nutrients and substrates found in stoat uterine secretions during diapause and/or the co-culture of blastocysts on uterine cell layers may better support the survival and development of expanded blastocysts *in vitro*.

Findings from this study and an earlier report (O'Connor et al. 2006) confirm that most wild-caught females that are brought into captivity in January carry diapausing blastocysts that survive for at least 24 weeks *in utero*. This means that until a method is established for culturing these *in vitro*, whole-animal trials remain the best method for testing fertility control agents targeting diapause, provided that methods can be developed to distinguish between pregnant and non-pregnant females. Faecal hormone monitoring may be one method of discrimination and results of research on this method will

be presented as part of a subsequent report. An alternate approach is to revisit the use of imaging techniques such as ultrasound and CAT scanning (O'Connor et al. 2006). Stoat blastocysts grow up to 1 mm during diapause (Amstislavsky et al. 1993, 1994) and recent technological advances in image resolution now permit detection of structures as small as 0.5 mm.

6. Conclusions

- Significant progress has been made in the development of artificial reproductive technologies to control and manipulate stoat reproduction in captivity. For the first time, methods have been established for the liquid (short-term) and frozen (long-term) storage of stoat sperm.
- The biological competence of frozen-thawed stoat sperm has been validated *in vitro*, based on the motility and acrosome integrity of sperm after culture.
- A non-surgical artificial insemination technique is in development for stoats, but its success is reliant on developing alternative methods of inducing ovulation.
- Treatment of females outside the breeding season with PMSG did not reliably stimulate ovarian follicle development and failed to induce ovulation. Ovulated eggs and embryos were only recovered after mating oestrous females.
- Treatment of oestrous females with PMSG did not improve the reliability and yield of eggs and embryos recovered after natural mating, or their capacity to develop *in vitro* compared with untreated controls.
- *In vitro* culture and the development of *in vivo* derived one- to four-cell embryos to the blastocyst stage (with up to 8% of these hatching) has been achieved for the first time in stoats. This culture system did not support the viability of diapausing blastocysts *in vitro*, suggesting that alternative substrates, nutrients and/or co-culture with uterine cell layers are required.

7. Recommendations

The authors recommend that:

- Further research is needed to develop methods of inducing ovulation for establishing artificial insemination technology, especially using preserved stoat sperm. This would provide a cheaper and more reliable alternative to natural mating for achieving pregnancy and for testing the ability of fertility control agents to disrupt egg development and fertilisation.

- Stoat researchers should adopt and refine the *in vitro* embryo culture system developed in this project to evaluate techniques to enhance stoat reproduction, and as a cost-effective alternative to whole-animal trials for testing fertility control agents targeting early embryo development.
- Further research is needed to establish a method to distinguish between pregnant and non-pregnant females during diapause, to support whole-animal trials for testing fertility control agents targeting diapause.

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How can we manipulate stoat reproduction?

Artificial reproductive technologies are being developed for stoats in New Zealand to better understand and manipulate stoat reproduction. Methods were established for liquid (short-term) and frozen (long-term) storage of stoat sperm. Frozen-thawed sperm maintained motility and acrosome integrity after in vitro *culture. A non-surgical artificial insemination technique is being developed to confirm that these sperm are capable of fertilising eggs* in vivo*. For the first time, an* in vitro *culture system that supports development of* in vivo *derived early-stage embryos to the blastocyst stage was achieved in stoats. These findings will enhance captive breeding of stoats for research, and future testing and prioritisation of reproduction-based targets for fertility control.*

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