Induction of ovulation and natural oestrous cycling in the Stripe-faced Dunnart, *Sminthopsis macroura*

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Abstract

Induced ovulation allows reproduction by otherwise infertile females, and is ideal for the captive breeding of endangered species where the population is aged or breeding is unsuccessful. A predictable time of ovulation after induction has not yet been achieved in polyovular marsupials. Ovulation was induced in *Sminthopsis macroura* using an initial injection of 20 IU equine serum gonadotrophin (eSG; Day 0), followed on Day 4 by either 20 IU eSG ($n=25$) or 0.5 mg porcine luteinizing hormone ($n=26$). I.p. hormone injection was given in the morning or early evening, and reproductive status was established prior to induction. Five non-cyclic animals began to cycle naturally following induction and one gave birth to a litter. The time of ovulation after the 1st injection (7.8 ± 0.9 days) was significantly shorter ($P=0.000$) and less variable than the previous study, mimicked the timing of natural cycling, and both natural and induced animals ovulated in the early morning. *In vitro* oocyte movement through the oviduct, observed for the first time in a marsupial, occurred in pulses. We estimated one group of oocytes could travel the length of the oviduct in 40 min, but it was probably around 4 h. The entire ovulation time (including multiple ovulations) was estimated at 7.5 h. This study has achieved a predictable timing of ovulation after stimulation, and induced non-cyclic animals to cycle naturally and give birth, providing a modified methodology for use in captive breeding programs of endangered dasyurid marsupial species with low fecundity.

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Introduction

Manipulation of ovulation allows the captive breeding of both endangered and laboratory species to be optimised. It allows the use of infertile animals, whether seasonal or physiological, increasing the productivity of the colony and aiding the collection of reliably timed samples. In Australia, more than 30% (18 of 55 species) of all dasyurids (Menkhorst & Knight 2004) are listed as threatened (Department of Environment and Heritage 2006, EPBC Act List of Threatened Fauna; Commonwealth of Australia, www.deh.gov.au), and a successful captive breeding program, including a reliable induced ovulation protocol, is essential.

Ovulation has been induced in all marsupial species studied, and although a behavioural oestrus is quite difficult to induce, it has been observed in *Macropus eugenii*, *Monodelphis domestica*, *Sminthopsis crassicaudata* (Hinds *et al.* 1996) and *Sminthopsis macroura* (Hickford *et al.* 2001). The current challenge for polyovular marsupials is obtaining a predictable timing of ovulation following induction.

Equine serum gonadotrophin (eSG), which functions with both follicle stimulating hormone (FSH) and luteinizing hormone (LH)-like activity (Hinds *et al.* 1996), has been used to stimulate follicle development in many marsupial species. In Dunnarts (*S. crassicaudata* (Rodger *et al.* 1992) and *S. macroura* (Hickford *et al.* 2001)), a single dose induces ovulation, however, in *S. macroura*, negative side-effects were observed, including nausea and increased numbers of luteinized follicles (LF; Hickford *et al.* 2001). A measure of 0.087 IU/g eSG given as two doses, does not have these problems, but results in an unpredictable time of ovulation (3–16 days after initial injection), with only 41% of animals ovulating by Day 9 post-injection (Hickford *et al.* 2001). The range of ovulation times is not given for *S. crassicaudata*, in which 66% had ovulated by Day 6 post-injection (Rodger *et al.* 1992).

The time taken by oocytes for oviduct transit has not been experimentally confirmed in any marsupial species. Available data (Tyndale-Biscoe & Renfree 1987, Selwood & Woolley 1991, Breed & Leigh 1992), and the onset of cleavage after uterine entry in all species

(Tyndale-Biscoe & Renfree 1987) suggests that it is probably <24 h in marsupials, significantly shorter than the 3–4 days that oocytes and conceptuses remain in the eutherian oviduct (Harper 1988).

S. macroura is a polyoestrous, polyovular, seasonally breeding dasyurid marsupial (Woolley 1990). The oestrous cycle length is 23.25 days (Woolley 1990). Day 0 of the oestrous cycle is defined as the day before the first day of body weight rise, indicating the beginning of the follicular phase (Selwood & Woolley 1991). Oestrus occurs after 3–4 days and spontaneous ovulation terminates the follicular phase after 6–7 days (Selwood & Woolley 1991).

In order to get a more predictable response following induced ovulation, the timing of the two hormone injections was modified to more closely replicate the natural hormonal stimulus on the ovary. The time between the two injections was reduced from 5 days (Hickford *et al.* 2001) to 3.75 or 4 days, which is when oestrus begins, and both morning and evening injection times were tested. Porcine LH was used for the first time in a polyovular marsupial and we also present the first *in vitro* investigation of oviducal transit by oocytes in a marsupial.

Materials and Methods

Animals

A laboratory colony maintained at The University of Melbourne by LS, following Australian National Health and Medical Research Council Guidelines for the Care and Use of Animals for Scientific Purposes, provided the 92 *S. macroura* females used in this study.

Daily monitoring of urine and body weight as previously described in Hickford *et al.* (2001), identified oestrous cycles. The follicular phase is 6–7 days long and is indicated by a steady rise in animal weight associated with increased cornified epithelial cell number in the urine. A weight drop associated with high levels of polymorphonuclear leucocytes in the urine sample indicates ovulation has occurred (Selwood & Woolley 1991) and is deemed time 0 of gestation (Selwood & Hickford 1999). Oviduct passage time is estimated as 8 h (Selwood & Woolley 1991). The luteal phase, which is contained within the gestation phase, begins the following morning on Day 1 and is maintained until Day 9 of gestation (Selwood & Woolley 1991). The gestation phase is only 10.7 days long (Selwood & Woolley 1991), the shortest of any mammal. A sharp drop in body weight, accompanied by yellow crystals in urine, a clear secretion in the pouch, and pouch young if pregnant, ends the gestation phase. The time between the end of these changes in a non-pregnant cycle and the beginning of the next follicular phase is termed the intermediate phase.

Animals were grouped according to their reproductive phase at initial stimulation: non-cyclic, or the follicular, gestation and intermediate phase of the oestrous cycle. Stimulation during the middle of the gestation phase is

generally unsuccessful, probably due to high ovarian progesterone levels (Hickford *et al.* 2001), so in this study stimulation only occurred early or late in the gestation phase, when corpora lutea (CL) influence was minimal.

Treatments

The day of the 1st injection was designated as Day 0 and the female was monitored daily until killed between Days 6 and 15. A male was placed in with the female on Day 4, and if mating was not observed, a new male was introduced on Days 5 and 6. Naturally cycling females were killed between Days 6 and 8 of the oestrous cycle with Day 0 of the natural cycle being the day before the first day of weight rise. Five females identified as non-cyclic were induced to ovulate using both eSG/eSG ($n=2$) and eSG/LH ($n=3$), and then allowed to cycle naturally once ($n=3$) or twice ($n=2$), before they were killed between Days 6 and 8 of the subsequent natural oestrous cycle unless pouch young were born.

Previous induced ovulation protocols have calculated the hormonal dose based on animal weight. Here, we have attempted to simplify the procedure by giving a constant dose to all animals. An initial injection of 20 IU eSG (Folligon, Allhank Trading Co., South Melbourne, Vic, Australia) was given in 100 μ L calcium- and magnesium-free PBS at either 10:00 or 18:00 h on Day 0. A subsequent injection of either 20 IU eSG or 0.5 mg porcine LH, also in 100 μ L PBS (a gift from Bioniche Animal Health, Armidale, NSW, Australia), was given at 10:00 or 18:00 h on Day 4. The diluted hormone was hand-warmed before i.p. injection just above the pouch using a 1.0 mL sterile syringe with a 30-gauge needle.

The interval between the 1st and 2nd injection varied from 3.75 days (18:00 Day 0 to 10:00 Day 4 – evening/morning (E/M)), to 4 days (10:00 Day 0 to 10:00 Day 4 – morning/morning (M/M); or 18:00 Day 0 to 18:00 Day 4 – evening/evening (E/E)).

Analysis

All experimentation began between 07:00 and 10:00 h to target ovulation. *S. macroura* is nocturnal, so it was assumed the ovulation time would be similar to other nocturnal animals, such as rats (Pederson 1951, Austin & Braden 1954) and mice (Runner 1947). This assumption was supported by the fact that oviducal oocytes and conceptuses were most regularly found in the morning (Selwood & Woolley 1991). Females were killed by inhalation of Halothane (Rhone Merieux, West Footscray, Vic, Australia) followed by cervical dislocation. The reproductive tract was removed and washed in warmed (35 °C) PBS. The ovary was examined under a dissecting microscope (Zeiss, North Ryde, NSW, Australia) to confirm the oestrous cycle stage and to identify the number, presence and size of Graafian follicles (GF) and

CL. The GF were examined to identify LF or atretic follicles (AF). Ovaries from animals allowed to cycle naturally for one or two cycles after induction were examined for the number of corpora albicans (CA) present. CA can identify how many oocytes were ovulated in the previous cycle (Woolley 1990). The transparent oviducts were carefully examined under transmitted light to identify any oocytes. The uteri were slit medially and any contents were analysed using the criteria from Selwood & McCallum (1987) to assess fertilization and normal development. The developmental stage of GF, CL and uterine contents were used to estimate the time of ovulation after the first injection, except in over-stimulated animals with AF and LF present.

Time of oviducal transit

Reproductive tracts found with oocytes in the oviduct, were placed whole into a sterile 35-mm Petri dish (Greiner, Interpath Services, West Heidelberg, Vic, Australia) containing Hepes Buffered Dulbecco's Modified Eagles Medium (HDMEM, Sigma-Aldrich, Castle Hill, NSW, Australia) supplemented with 10% Foetal Calf Serum (Sigma-Aldrich) and held at 35 °C. Initial trials, in which the oviduct was straightened and affixed to wax, or in which the oviducts were held in continuous white light, resulted in the cessation of peristalsis within 10–15 min. Therefore, the oviduct was left attached to the ovary and uterus, and in between observations was covered by a box. The position of the oocytes in the oviduct was noted every 10 min for up to 1 h, and after this, at intervals of 1 h or less. Brief observations (2 min) of oocyte position were made under transmitted white light using a dissecting microscope with a heated stand. The oocytes were grouped according to the region of the oviduct that they were found: upper and lower ampulla, and upper and lower isthmus. Once the oviducts ceased peristalsis, or if the oocytes had not moved for more than 2 h, the experiment ceased.

Statistics

The proportion of animals induced, identified by the presence of ovarian stimulatory products (GF or CL), was calculated from all animals given the hormonal stimulation. The proportion of animals showing mating success, identified from observed behaviour and/or the presence of sperm in morning urine samples, was calculated as the proportion of induced animals that mated when paired with a male. The proportion of animals showing ovulation success was calculated as the proportion of animals examined after Day 7 that had ovulated. The proportion of animals showing fertilization success was calculated from animals that were found with zygotes that had been paired with a male. The proportion of animals that were stimulated, mated, ovulated and fertilized was compared

with the reproductive phase of initial stimulation, and induction hormone and time of injection using a χ^2 -test. The number of CL/GF, LF, AF, ovulated oocytes and conceptuses were calculated as the mean $\pm$ s.d. for each group and analyzed using one-way ANOVA followed by Bonferroni's *post-hoc* test to identify significant differences at $P < 0.01$. The time of ovulation (pre- and post- the time of experimentation) was estimated from daily monitoring, ovarian appearance (GF and CL), oviducal appearance and (if present) conceptus development. To compare the time of ovulation after induction to the previous study, a one-sample *t*-test using the mean value from Hickford *et al.* (2001) as the test value was used, and an independent sample *t*-test compared the time of ovulation between induced and natural cycles. Two-way ANOVA compared the time of ovulation after initial injection for the two hormones given and the time of hormone injection. The level of significance, except for Bonferroni's *post-hoc* test, was $P \leq 0.05$.

Results

Effect of the reproductive phase of initial injection

The previous study (Hickford *et al.* 2001) found that the proportion of animals induced during the middle of the gestation phase was lower than those induced during the other reproductive phases. In this study, animals were not induced during the middle of the gestation phase, and with the exception of increased LF in non-cyclic animals (Table 1; $P = 0.003$), no significant difference was found in the number of CL and GF, AF, ovulated oocytes and conceptuses (Table 1) between the four reproductive phases. The time to ovulation after induction (Table 2) and the ovarian stimulation, mating, ovulation or fertilization success (Table 3) were also no different between the four reproductive phases. Therefore, the four reproductive phases have been pooled to evaluate the influence of the two hormone protocols and the three time of daily injection variables.

Time to ovulation

The time to ovulation after induction (Table 4; 7.8 ± 0.9 days) was significantly shorter ($P = 0.000$) and less variable than in the previous study (Hickford *et al.* 2001) and significantly longer than naturally cycling animals (Table 4; $P = 0.033$). There was no difference in the timing of ovulation after induction using eSG/eSG or eSG/LH, or the three time protocols (Table 4).

Induction success

A significantly lower proportion of animals induced using eSG/eSG in two evening injections (E/E) showed ovarian stimulation (Table 5; $P = 0.034$) compared to

Table 1 Number of stimulatory products (per animal) found in *Sminthopsis macroura* that responded to the induced ovulation protocol during the four reproductive phases.

Reproductive phase stimulated	n	Stimulatory products (number per animal (mean $\pm$ S.D.))				
		Corpora lutea or Graafian follicles (n) ^a	Luteinized follicles	Atretic follicles	Ovulated oocytes (n) ^b	Conceptuses (n) ^c
Non-cyclic	7	29.0 $\pm$ 16.6 (5)	15.3 $\pm$ 21.0*	0.1 $\pm$ 0.4	26.5 $\pm$ 7.8 (2)	0.0 $\pm$ 0.0 (2)
Follicular	17	35.5 $\pm$ 14.3 (13)	3.7 $\pm$ 8.1	5.2 $\pm$ 11.0	15.5 $\pm$ 11.9 (10)	0.0 $\pm$ 0.0 (3)
Gestation	11	39.6 $\pm$ 18.5 (11)	2.4 $\pm$ 6.6	3.6 $\pm$ 6.0	23.9 $\pm$ 12.8 (8)	2.4 $\pm$ 3.2 (5)
Intermediate	13	29.3 $\pm$ 14.1 (12)	0.5 $\pm$ 1.9	4.3 $\pm$ 6.1	21.8 $\pm$ 16.5 (11)	13.1 $\pm$ 12.1 (7)
ANOVA		$F_{3,38}$ 1.030	$F_{3,44}$ 4.006	$F_{3,44}$ 0.694	$F_{3,27}$ 0.750	$F_{3,13}$ 2.822
P value		0.391	0.003*	0.561	0.532	0.080

*Indicates significant difference from other reproductive phases. P value level of significance is <0.01 (Bonferroni's adjustment). Sample size (n) indicates the number of animals stimulated. Animals that were not induced were not included in this table.

^aAnimals were excluded from analysis of the means if they were overstimulated. ^bAnimals were excluded from analysis of the means if they did not ovulate. ^cAnimals were excluded from analysis of the means if they were not paired with a male or did not ovulate.

M/M or E/M protocols. However, the proportion of all induced animals that responded to the induction protocol (Table 5) was not significantly different from natural cycles.

The proportion of induced animals identified as having mated after being paired with a male (Table 5) was significantly higher than identified in natural cycles ($P=0.037$), but in this study a successful mating was more likely to be identified during an induced cycle. Eight of nine induced animals (88.9%) found with conceptuses were previously identified as having mated, but only two of nine (22.2%) naturally cycling animals found with conceptuses were identified (χ^2 5.625, $P=0.018$). The hormonal protocol and the time of injection did not influence the likelihood of an animal mating (Table 5).

The proportion of all induced animals that successfully ovulated (Table 5) was significantly lower than natural cycles ($P=0.042$). No significant difference in the number of animals that ovulated was found between the two hormonal protocols, or the time of injection (Table 5).

The proportion of animals that had conceptuses (Table 5) was the same between induced and natural cycles. No animals induced using E/E injections were included in this analysis because all post-ovulation E/E experiments occurred when oocytes were still in the oviduct. There was no significant difference in the number of animals with conceptuses between the two hormonal protocols, or the time of injection (Table 5).

Induction products

There were significantly more CL/GF (Table 6; $P=0.000$), ovulated oocytes (Table 6; $P=0.007$) and AF (Table 6; $P=0.009$) found in induced cycles than occurred in natural cycles. The number of conceptuses found after E/M protocols was significantly higher in eSG/LH animals than in eSG/eSG animals (Table 6; $P=0.003$). No other differences in the products of induction were observed between the two hormonal protocols or the three time of injection protocols (Table 6).

Induction of natural cycles

One previously non-cyclic female gave birth to four pouch young in her first natural cycle following induced ovulation. The other four animals allowed to cycle naturally after induction showed normal follicular development and ovulation when examined during the first ($n=2$) or second natural cycle ($n=2$). The number of CA found was significantly higher in animals where the previous cycle was the induced cycle than those which were undergoing their second natural cycle (1st 34.0 ± 5.7 , $n=2$; 2nd 15.5 ± 2.1 , $n=2$; $t=4.331$, df 2, $P=0.049$), showing that although the number of CL/GF was significantly higher in induced cycles, subsequent natural cycles return to normal levels. There was no difference in the number of products during oestrus or following ovulation in the induced natural cycles, and both the induced and natural cycles (Table 6), probably due to the low sample size. Two of five animals mated during the first natural cycle after induction, and one of these matings resulted in a litter. One of two animals mated during the second natural cycle, and this mating resulted in 17 fertilized conceptuses.

Oviducal transit

Animals found with oviducal oocytes exhibited oviducal peristalsis and the infundibulum completely enveloped

Table 2 The time to ovulation (days after first injection) in *Sminthopsis macroura* induced to ovulate during the four reproductive phases.

Reproductive phase stimulated	n	Time to ovulation (in days after 1st injection)	
		Mean $\pm$ S.D.	Min, max
Non-cyclic	5	8.3 $\pm$ 1.0	7.0, 9.0
Follicular	13	7.8 $\pm$ 0.9	6.5, 9.0
Gestation	10	7.8 $\pm$ 0.9	7.0, 9.5
Intermediate	12	7.7 $\pm$ 1.2	5.0, 9.0
ANOVA		$F_{3,36}$ 0.488, $P=0.693$	

Animals that were not induced, or were overstimulated (as determined by follicle appearance) and therefore unable to ovulate, were not included in this table.

Table 3 Comparison of the proportion of *Sminthopsis macroura* that showed ovarian stimulation, mated, ovulated and, if paired and ovulated, had conceptuses between the four reproductive phases of initial stimulation.

Reproductive phase stimulated	Responders/total examined			
	Induced ^a	Mated ^b	Ovulated ^c	With conceptuses ^d
Non-cyclic	7/7	2/7	2/5	0/2
Follicular	17/18	5/12	11/16	0/3
Gestation	11/12	4/7	8/10	3/6
Intermediate	13/14	6/10	11/12	6/8
χ^2	0.611	2.057	5.420	0.143
<i>P</i> value	0.894	0.561	0.143	0.073

χ^2 – chi-square analysis.

^aAll animals that received hormonal injections were included in this analysis of responders. ^bOnly animals that were induced and that were paired with a male were included in this analysis of responders. ^cOnly animals that were induced and were examined from Day 7 were included in this analysis of responders. ^dOnly animals that were induced, were paired with a male and ovulated were included in this analysis of responders.

the ovary. Maintenance of peristalsis for up to 18 h *in vitro* required the whole reproductive tract, especially with intact connective tissue from the oviduct to the ovary and uterus, to be held in HDMEM in the dark. Despite continual peristalsis *in vitro*, oocytes were generally static, moving down the oviduct in 'pulses' ($n=6$ in 3 animals). During a 'pulse', a group (2.8 ± 0.9 , $n=6$) of oocytes were quickly pushed along 1/4 of the length of the oviduct in as little as 10 min ($n=3$). The first movement of oocytes occurred 30, 45, and 90 min after the beginning of culture. Unfortunately, no oocyte group moved more than once during the culture period, so we

Table 4 The time to ovulation in *Sminthopsis macroura* induced to ovulate with eSG/eSG or eSG/LH given in morning or evening injections.

Hormone (injection time)	Time to ovulation (in days after 1st injection)		
	<i>n</i>	Mean $\pm$ s.d.	Min, max
eSG/eSG			
M/M	11	8.5 ± 0.7	7.0, 9.0
E/M	6	7.4 ± 0.9	6.5, 9.0
E/E	2	7.5 ± 0.0	7.5, 7.5
eSG/LH			
M/M	14	7.6 ± 1.1	5.0, 9.5
E/M	5	7.6 ± 0.8	7.0, 9.0
E/E	2	8.0 ± 0.7	7.5, 8.0
Two-way ANOVA		$F_{6,37} 2.054$	$P=0.083$
Total induced	40	$7.8 \pm 0.9^{*†}$	5.0, 9.5
Hickford <i>et al.</i> (2001)	22	9.4 ± 3.9	3.0, 16.0
Total vs Hickford <i>et al.</i>		$t=10.188$	$P=0.000^{*}$
Natural	8	7.0 ± 1.1	5.0, 9.0
Total vs natural		$t=2.194$	$P=0.033^{†}$

Animals that were not induced, or were overstimulated (as determined by follicle appearance) and therefore unable to ovulate, were not included in this table. ^{*}Indicates significant difference from Hickford *et al.* (2001). [†]Indicates significant difference from natural cycles. eSG, equine serum gonadotrophin; LH, luteinizing hormone; M/M, morning/morning injections; E/M, evening/morning injections; E/E, evening/evening injections.

Table 5 Comparison of the proportion of *Sminthopsis macroura* that showed ovarian stimulation, mated, ovulated and had conceptuses after induced ovulation with eSG/eSG or eSG/LH given in morning or evening injections.

Induction hormone (injection time)	Responders/total examined			
	Induced ^a	Mated ^b	Ovulated ^c	With conceptuses ^d
eSG/eSG				
M/M	14/14	4/11	8/12	4/6
E/M	6/6	2/6	5/6	1/5
E/E	3/5*	3/3	2/3	na
χ^2	8.696	4.328	0.328	1.742
<i>P</i> value	0.034*	0.115	0.849	0.187
eSG/LH				
M/M	15/16	4/9	11/13	4/9
E/M	5/5	1/2	4/5	2/2
E/E	5/5	3/5	1/4	na
χ^2	0.650	0.311	5.653	2.037
<i>P</i> value	0.723	0.856	0.059	0.154
eSG/eSG vs eSG/LH				
M/M χ^2	0.905, $P=0.341$	0.135, $P=0.714$	0.361, $P=0.548$	0.444, $P=0.505$
E/M χ^2	0.000, $P=1.000$	0.178, $P=0.673$	0.020, $P=0.887$	3.733, $P=0.053$
E/E χ^2	2.500, $P=0.114$	1.600, $P=0.206$	1.215, $P=0.270$	na
Total induced	48/51	17/36 [†]	31/43 [†]	11/19
Natural	32/32	2/16	26/27	9/15
χ^2	1.953	5.759	5.589	0.537
<i>P</i> value	0.162	0.037 [†]	0.042 [†]	0.464

^{*}Indicates significant difference from other injection times using the same hormone protocol by χ^2 (chi-square) analysis. [†]Indicates significant difference from natural cycles using χ^2 analysis.

^aAll animals that received injections were included in the analysis of responders. ^bOnly animals that were induced and that were paired with a male were included in this analysis of responders. ^cOnly animals that were induced and were examined from Day 7 were included in this analysis of responders. ^dOnly animals that were induced, were paired with a male and ovulated were included in this analysis of responders. eSG, equine serum gonadotrophin; LH, luteinizing hormone; M/M, morning/morning injections; E/M, evening/morning injections; E/E, evening/evening injections; na, not available.

estimate that a 1 h long period of stasis between oocyte movements is likely, given the range of times for first movement after culture initiation. Ovulation was observed four times (from three animals) *in vitro*, and occurred at 30, 45 min, 4 and 5 h after the beginning of culture. The appearance of oocytes in the upper ampulla after ovulation took <10 min in one event, and <30 min in all observed events. One animal ovulated twice in culture, and the second ovulation occurred within 1 h of the first. The discrete placement of oocyte groups found spaced along the oviduct and in the uterus of two animals and the separate ovulation events observed during culture, indicate that more than one ovulation 'event' occurred for most animals. The maximum number of ovulation events suggested by oocyte placement and observed ovulations was four, but the average was $1.8 (\pm 1.3, n=5)$. Ovulation was not synchronous between the ovaries, with three of five

Table 6 The number of corpora lutea or Graafian follicles, luteinized follicles, atretic follicles, ovulated oocytes and conceptuses, found in *Sminthopsis macroura* induced to ovulate using eSG/eSG or eSG/LH given in morning or evening injections.

Induction hormone (injection time)	Number per animal (mean $\pm$ s.d.)				
	Corpora lutea or Graafian follicles (n) ^a	Luteinized follicles (n)	Atretic follicles (n)	Ovulated oocytes (n) ^b	Conceptuses (n) ^c
<i>eSG/eSG</i>					
M/M	25.6 $\pm$ 13.3 (13)	1.4 $\pm$ 3.7 (14)	2.4 $\pm$ 5.5 (14)	20.6 $\pm$ 15.7 (7)	13.7 $\pm$ 18.0 (3)
E/M	40.0 $\pm$ 14.7 (5)	4.7 $\pm$ 11.4 (5)	1.8 $\pm$ 2.5 (5)	19.6 $\pm$ 7.5 (5)	0.4 $\pm$ 0.9 (5)*
E/E	41.6 $\pm$ 11.0 (3)	17.7 $\pm$ 30.6 (3)	0.0 $\pm$ 0.0 (3)	20.5 $\pm$ 3.5 (2)	na
ANOVA	$F_{2,18}$ 3.131	$F_{2,20}$ 2.432	$F_{2,19}$ 0.339	$F_{2,12}$ 0.040	$F_{1,6}$ 3.056
<i>P</i> value	0.068	0.113	0.716	0.961	0.131
<i>eSG/LH</i>					
M/M	35.1 $\pm$ 17.9 (15)	1.9 $\pm$ 5.8 (15)	5.3 $\pm$ 9.2 (15)	22.9 $\pm$ 17.0 (11)	5.3 $\pm$ 8.5 (7)
E/M	32.6 $\pm$ 11.5 (5)	0.8 $\pm$ 1.9 (5)	6.2 $\pm$ 7.8 (5)	24.3 $\pm$ 9.6 (4)	12.0 $\pm$ 5.7 (2)*
E/E	61.0 $\pm$ (1)	11.0 $\pm$ 15.1 (5)	6.4 $\pm$ 14.3 (5)	7.0 $\pm$ (1)	na
ANOVA	$F_{2,18}$ 1.243	$F_{2,22}$ 2.782	$F_{2,22}$ 0.032	$F_{2,13}$ 0.521	$F_{1,7}$ 1.046
<i>P</i> value	0.312	0.084	0.969	0.606	0.341
<i>eSG/eSG</i> vs <i>eSG/LH</i>					
M/M <i>t</i> -test	<i>t</i> 1.577	<i>t</i> 0.256	<i>t</i> 0.956	<i>t</i> 0.342	<i>t</i> 1.044
<i>P</i> value	0.268	0.800	0.347	0.735	0.327
E/M <i>t</i> -test	<i>t</i> 0.885	<i>t</i> 0.7429	<i>t</i> 1.203	<i>t</i> 0.029	<i>t</i> 5.225
<i>P</i> value	0.402	0.477	0.263	0.978	0.003*
E/E <i>t</i> -test	<i>t</i> 1.520	<i>t</i> 0.424	<i>t</i> 0.750	<i>t</i> 1.607	na
<i>P</i> value	0.268	0.751	0.482	0.152	
Total induced	33.5 $\pm$ 15.8 (42) [†]	3.9 $\pm$ 10.4 (48)	3.9 $\pm$ 7.9 (48) [†]	22.8 $\pm$ 12.5 (28) [†]	6.1 $\pm$ 9.8 (17)
Natural	20.0 $\pm$ 8.72 (26)	0.7 $\pm$ 3.7 (24)	0.6 $\pm$ 2.3 (24)	14.6 $\pm$ 8.7 (26)	7.5 $\pm$ 10.0 (14)
<i>t</i> -Test	3.999	1.881	2.710	2.784	0.388
<i>P</i> value	0.000 [†]	0.065	0.009 [†]	0.007 [†]	0.701
Induced natural cycle vs Induced	18.0 $\pm$ 1.4 (4)	0.0 $\pm$ 0.0 (4)	1.3 $\pm$ 1.9 (4)	16.5 $\pm$ 0.7 (2)	8.5 $\pm$ 12.0 (2)
<i>t</i> -test	0.735	0.672	1.945	0.186	0.694
<i>P</i> value	0.466	0.505	0.058	0.853	0.552
vs Natural					
<i>t</i> -test	0.402	0.555	0.451	0.613	0.610
<i>P</i> value	0.691	0.584	0.656	0.544	0.548

*Indicates significant difference between the two hormonal protocols in E/M animals. [†]Indicates significant difference from natural cycles. *P* value level of significance is <0.01 (Bonferroni's adjustment). Sample size (*n*) indicates the number of animals stimulated. Animals that were not induced were not included in this table.

^aAnimals were excluded from analysis of the means if they were overstimulated. ^bAnimals were excluded from analysis of the means if they did not ovulate. ^cAnimals were excluded from analysis of the means if they were not paired with a male or did not ovulate. eSG, equine serum gonadotrophin; LH, luteinizing hormone; M/M, morning/morning injections; E/M, evening/morning injections; E/E, evening/evening injections; na, not available.

animals showing asynchronous ovulations based on initial oocyte position in the oviduct and uterus at the beginning of the culture.

Discussion

Non-cyclic animals that were induced to ovulate began cycling naturally, showing follicular development, mating, ovulation and fertilization. The birth of a litter from a natural cycle following induced ovulation makes this protocol promising for the captive breeding of dasyurids with asynchronous breeding. Non-cyclic females can be induced to ovulate and then to cycle naturally, allowing pairing with breeding males.

The timing of induced ovulation was highly predictable using this protocol. Both induced and natural ovulations occurred before 07:00 h, with oocytes found in the oviduct in the early morning. This photoperiod constraint on the timing of ovulation is reflected in our

s.d. (± 1.0 days). The day of ovulation in mammals is regulated by increasing ovarian oestradiol secretion (Johnson & Everitt 2000) and the time of day of ovulation is regulated by a stimulus from the suprachiasmatic nuclei in the hypothalamus, which induces the LH surge from the anterior pituitary (Kawakami *et al.* 1980, Gajewska *et al.* 2005). Interestingly, in this study, there was no difference in the time of ovulation after morning or evening LH stimulation. Our results suggest that there is a third tier of control, which prevents ovulation if the LH surge occurs at the wrong time of day. Recent studies have identified a circadian rhythm in the expression of two clock genes, *per1* and *per2*, in steroidogenic cells of the rat ovary (Fahrenkrug *et al.* 2006), which may regulate intraovarian processes leading to ovulation.

The size and degree that the infundibulum envelopes the ovary indicates the closeness to the time of ovulation *in vivo* (Woolley 1990). In this *in vitro* study, one infundibulum was observed to retreat slightly within 1 h

of the last *in vitro* ovulation, supporting our conclusion that oviducal oocytes were recently ovulated. This study is the first *in vitro* investigation into the time taken by oocytes to travel through the marsupial oviduct. *In vitro* oocyte movement occurred in pulses, which is also observed in rabbits (Bourdage & Halbert 1984). However, if oocyte movement is continuous *in vivo*, our results suggest that it could take as little as 40 min for one group of oocytes to travel the length of the oviduct, from the upper ampulla to uterine entry. If the stasis observed is natural, we suggest that a 1-h period of stasis occurs after each movement. Thus, one group of oocytes would take about 4 h to move through the oviduct. Staggered ovulation events occurred *in vivo* and *in vitro*, both in time, with discrete groups of oocytes found spaced through the oviducts and between ovaries. *In vitro*, two ovulation events from the same ovary occurred only in one animal, 1-h apart and oocytes moved into the upper ampulla within as little as 10 min. In polyovular *S. macroura*, multiple ovulation events occur naturally (Selwood & Woolley 1991), explaining the short time between ovulation events found in this study compared to the monovular *Trichosurus vulpecula* (Glazier 1999) which had 2 ovulations over 6 h *in vivo* and *M. eugenii* (Molinia *et al.* 1998), which had 9 ovulations over 9 h *in vivo*. A maximum of four ovulation events in the one animal occurred in this study. In mice (Braden 1957) and pigs (Pope *et al.* 1988) all ovulations occurred within 3 h, however, it is unclear if they occur singly or in groups. We estimate that the total ovulation time (including four ovulation events) in *S. macroura* could take as little as 4 h if stasis does not occur *in vivo*, or 7.5 h if the oocytes remain static for 1 h in each region of the oviduct. Selwood & Woolley (1991) estimated total oviducal transit time in *S. macroura* was between 7 and 12 h and our findings suggest that it could be less than that. The transit of oviducal oocytes in pulses was seen here and in rabbits (Bourdage & Halbert 1984), both species with significant contributions to egg investments made by the oviduct. Secretion by the oviducal epithelium is enhanced by the presence of an oocyte in *M. domestica* (Phillips & Fadem 1987), suggesting that pauses in oviducal transit may allow synthesis of secretory proteins by the oviducal epithelium prior to further transit. The rapid oviducal transit of marsupial oocytes compared to eutherian oocytes is possibly because the marsupial conceptus must be surrounded by a shell coat, which is secreted by the uterine glandular epithelium, before blastocyst formation (Moore & Taggart 1993, Renfree & Lewis 1996, Casey & Selwood 2003) to prevent dispersal of the blastocyst epithelium (Casey & Selwood 2003) after mucoid coat loss.

The previous induced ovulation study in *S. macroura* (Hickford *et al.* 2001) followed the protocol used in *S. crassicaudata* (Rodger *et al.* 1992), with equivalent results. Other induced ovulation studies in marsupials

have used similar hormones, concentrations and timing of injections, with broadly similar results in a diverse range of species (Hinds *et al.* 1996, Glazier & Molinia 1998, Molinia *et al.* 1998, Glazier 1999). Following these data, we suggest that our protocol, where the second hormone injection is given at the beginning of oestrus, when oocytes are beginning to mature, will successfully induce predictable ovulation and natural cycling in other dasyurid species. However, we believe a return to the use of body weight to calculate an appropriate hormone dosage would reduce the number of LF and AF found on the ovary, especially in non-cyclic animals.

Whilst captive breeding of dasyurid species has been well documented (Godfrey 1969, Woolley 1966, 1971), it is labour intensive. An induced ovulation protocol such as this one, allows breeding from older and non-cyclic females, as well as making the time of mating and ovulation more predictable, which is useful in populations with limited males or where the remaining animals show asynchronous breeding.

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