Induced ovulation mimics the time-table of natural development in the stripe-faced dunnart, *Sminthopsis macroura* and results in the birth of fertile young

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Abstract

Induced ovulation maximizes captive breeding success, increasing productivity and facilitating the contribution of otherwise infertile animals to the genetic pool. In marsupials, induced ovulation to produce fertile young is unknown. Here we present an induction protocol efficient in inducing non-cycling and non-reproductive females to cycle, mate, ovulate, and conceive. Ovulation was induced in *Sminthopsis macroura* using an initial injection of 0.06 IU equine serum gonadotropin (eSG)/g (time 0), followed on day 4 by 0.04 IU eSG/g. Using this induction regime, the timing of follicular and embryonic development mimics natural cycles and results in the birth of viable, fertile young. Response to induction is not significantly affected by animal age, making this protocol an effective conservation tool. We have established a time-table of development following induction, providing a source of precisely timed research material. This is the first induced ovulation protocol in any marsupial to result in demonstrated fertile offspring and to allow the reliable collection of known-age samples during both the follicular phase and the gestation period.

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Introduction

Induced ovulation maximizes captive breeding success, allowing increased productivity of domestic species, laboratory colonies, and the development of more effective breeding programs for endangered species. Although the reproductive patterns of many marsupials are well characterized (Tyndale-Biscoe & Renfree 1987), knowledge of the hormonal changes associated with mating, ovulation, and fertilization remains scarce (Hinds *et al.* 1996). As a result, the development of induced ovulation protocols for marsupial species has relied largely on proven eutherian treatments and application in marsupials has had varying success (Hinds *et al.* 1996).

The marsupial ovary is highly sensitive to treatment with eutherian gonadotropins (Rodger *et al.* 1992a, Hinds *et al.* 1996). Responses differ between species, and successful application requires species-specific examination and refinement. In general, the optimum dose required to induce ovulation in marsupials appears much lower than reported in eutherians. Numerous authors have reported ovarian abnormalities in marsupials following over stimulation with exogenous hormones (Harding 1969, Renfree *et al.* 1988, Rodger & Mate 1988, Molinia *et al.* 1998, Hickford *et al.* 2001,

Menkhorst *et al.* 2007). Over stimulation, associated with an elevated concentration of plasma estradiol (Renfree *et al.* 1988), may result in degenerative ovarian changes such as cysts, premature follicle luteinization, and oocyte retention (Rodger 1990).

Among the studied monovular marsupials, induced ovulation has been described in *Macropus eugenii* (Renfree *et al.* 1988, Magarey *et al.* 2003), *Trichosurus vulpecula* (Harding 1969, Rodger & Mate 1988, Glazier & Molinia 1998, 2002, Glazier 1999), and *Bettongia penicillata* (Hayman *et al.* 1990, Rodger *et al.* 1992b). Induced ovulation protocols have also been applied to polyovular marsupials including, *Monodelphis domestica* (Nelson & White 1941), *Dasyuroides byrnei* (Fletcher 1983), *Sminthopsis crassicaudata* (Smith & Godfrey 1970, Rodger *et al.* 1992a, Hinds *et al.* 1996, Mate 1998), and *Sminthopsis macroura* (Hickford *et al.* 2001, Menkhorst *et al.* 2007); however, an induction protocol that can result in normal embryos is rare, and a protocol that can result in the birth of normal and fertile pouch young (PY) from an induced ovulation cycle of previously non-cycling or non reproductive females has yet to be successfully established.

S. macroura is a polyestrous, polyovular, seasonally breeding dasyurid (Woolley 1990), found in arid and

semi-arid regions of northern and central Australia. It has an estrous cycle length of 23.25 days (Woolley 1990) and the shortest mean gestation period (10.7 days) of any known mammal (Selwood & Woolley 1991). In some cases where development of the corpora lutea occurs at a faster rate, the gestation period can be as short as 9.5 days (Selwood & Woolley 1991). In natural cycles, the beginning of the follicular phase of the estrous cycle (time 0) occurs 24 h before the first day of weight rise and/or the appearance of cornified epithelial cells in the urine. Estrus corresponds with days 4–6 of the follicular phase, and spontaneous ovulation occurs on day 7 (Selwood & Woolley 1991). Intensive reproductive monitoring of this species in our long-term colony has allowed accurate determination of estrous cycle stage, day of ovulation, gestation period, and stage of pregnancy, providing an animal model in which the timing of hormonal administration can be accurately controlled.

Successful induction has been demonstrated in *S. macroura* using a variety of different induction regimes and hormonal concentrations (Hickford *et al.* 2001, Menkhurst *et al.* 2007). In investigating appropriate dosages, time of dosage delivery, time of ovulation and oviducal transit time, Hickford *et al.* (2001) and Menkhurst *et al.* (2007) have established that there is no significant difference in induction results between non-cyclic, intermediate, follicular, and gestation phases of the estrous cycle, excluding the part of the luteal phase where progesterone concentration is high (Hickford *et al.* 2001, Menkhurst *et al.* 2007). Typically, equine serum gonadotropin (eSG), administered as two injections (Hickford *et al.* 2001, Menkhurst *et al.* 2007), successfully induced ovulation of more oocytes per ovary than did ovulation in animals undergoing natural cycles and if mated, conceptuses resulted. However, both Hickford *et al.* (2001) and Menkhurst *et al.* (2007) noted an increase in the occurrence of ovarian abnormalities, a scenario preferably avoided for the breeding of endangered animals. Following induction, the frequency of atretic or prematurely luteinized ovarian follicles appeared greater in the study by Menkhurst *et al.* (2007), who employed a hormone dose comparatively larger than Hickford *et al.* (2001). Neither of the previous *S. macroura* induced ovulation studies resulted in the birth of PY, although previous non-cycling females began to cycle naturally following induction and one gave birth to a litter in her first natural cycle following induced ovulation (Menkhurst *et al.* 2007). Rodger *et al.* (1992a) induced *S. crassicaudata* to produce PY, but the reproductive status of the female was unclear and the study did not demonstrate whether the offspring produced were fertile.

The aims of the current study were: i) to establish an induction protocol in *S. macroura* that was easy to apply and led to the provision of a time-table of

follicular and embryonic development to enable the collection of precisely timed research material and ii) to determine the fertility of offspring produced following induced ovulation.

Results

The 57 females induced to ovulate in this study fell into three reproductive categories, 26 were cycling and reproductive, 20 were cycling but non-reproductive, and 11 were non-cycling. Despite deliberately selecting animals with poor reproductive performance, 97% (55/57) of induced females responded to the induction protocol, with only two non-responders (one cycling and reproductive in its first breeding season; one non-cycling in its second breeding season). Of the animals examined after day 6 post injection, 90% (43/48) showed evidence of successful ovulation. The five animals that did not ovulate include the two non-responders and three animals in their first breeding season (two cycling and reproductive; one cycling but non-reproductive) that contained atretic follicles within their ovaries. Animal age had no significant effect on the ability of animals to begin cycling ($t_{(55)}=0.80$, $P=0.45$) and/or ovulate ($t_{(46)}=0.21$, $P=0.84$) following induction.

Follicle development

Ovarian follicle development was examined in nine induced animals during the second half of the follicular phase (days 4–6.7 post initial injection) and compared with normal development in naturally cycling animals, $n=29$ (Fig. 1). Following induction, developing follicles showed normal morphology, and follicle diameter measurements recorded were within the range expected for naturally cycling animals (Kress *et al.* 2001, Nation & Selwood 2009). Primordial, primary, and early secondary follicles were present in all examined ovaries. By day 4 post injection ($n=3$), ovaries contained late tertiary stage follicles, with antral pocketing evident among cells of the granulosa layer. Antral stage follicles, containing antral lacunae of varying size and germinal vesicle stage oocytes were found between days 5 and 6.7 post injection ($n=6$). Oocytes from the largest and most advanced antral follicles (examined 6.7 days post-injection) remained in meiotic arrest, with centrally concentrated cytoplasmic vesicles, and adherent cumulus cells. The most mature oocytes showed developing cytoplasmic polarity and the beginnings of perivitelline space formation; however, they had yet to undergo germinal vesicle breakdown, extrude the first polar body, and shed the cumulus layer, essential events which preclude ovulation.

In comparison to previous studies (Hickford *et al.* 2001, Menkhurst *et al.* 2007), the incidence of follicular abnormalities (premature follicular luteinization,

Figure 1 Diagrammatic comparison of the time-table of follicular and embryonic development in naturally cycling versus induced *Sminthopsis macroura*. Within the follicular phase, the grey rectangles describe the maturational state of follicular oocytes; immature/germinal vesicle (GV) stage oocytes or meiotically mature metaphase II (MII) stage oocytes. The presence of primary, secondary, tertiary, and antral ovarian follicles is indicated by solid (observed) and dashed (expected) lines. TO, fertilized tubal oocytes; UB, unilaminar blastocyst; BB, bilaminar blastocyst; TB, trilaminar blastocyst; PY, pouch young; * indicates that development was timed from ovulation.

follicular atrophy, and retained oocytes) was low in this study, although still significantly higher than in the ovaries of naturally cycling animals (Table 1).

Comparison of the number of developing antral follicles or corpora lutea in the ovaries of induced and naturally cycling animals revealed that the induction protocol significantly increased the number of oocytes selected for maturation and subsequent ovulation (Table 1).

Embryonic development

A total of 19 induced animals were used for the timely collection of conceptuses in order to compare the rate of embryonic development with conceptuses obtained

from natural cycles ($n=29$). Of these, ten were cycling and reproductive, seven were cycling but non-reproductive, and two were non-cycling.

Using induced ovulation, fertilized oocytes were detected in oviducts as early as 167 h (or at 7.0 days) after administration of the first injection. Uterine zygotes were detected at 7.2 days, 2-cell conceptuses at 7.6 days, 4-cell conceptuses at 7.8 days, 16-cell conceptuses at 8.6 days, and unilaminar blastocysts at 10.9 days (Fig. 1). Bilaminar blastocysts were detected at 7.25 days post ovulation (Fig. 1). All conceptuses were of normal morphology. Since ovulation occurs 7 days post first injection, when these analyses were compared with the timely collection of conceptuses from naturally

Table 1 Summary of the proportion of induced and naturally cycling *Sminthopsis macroura* used for the analysis of follicular, embryonic, and parthenogenetic development, pouch young (PY) production, and failed experiments; and a comparison of the number of corpora lutea or antral follicles and abnormal follicles between induced and naturally cycling animals.

Stage and type of follicles and conceptuses	Animals (<i>n</i>)	Reproductive status (<i>n</i>)			Mean $\pm$ S.E.M. corpora lutea or antral follicles per ovary	Mean $\pm$ S.E.M. abnormal follicles per ovary
		NC	CNR	CR		
Induced (<i>n</i> =57)						
Follicles	9	2	4	3	12.47 $\pm$ 0.61 ^a (<i>n</i> =43)	1.77 $\pm$ 0.29 ^b (<i>n</i> =43)
Embryos	19	2	7	10		
Parthenotes	15	3	3	9		
Pouch young	2	1	0	1		
Failed						
Responded and mated but no PY	1	0	1	0		
Responded but did not mate	6	2	4	0		
Failed						
Atretic	3	0	1	2		
Non-responsive	2	1	0	1		
Total		11	20	26		
Natural (<i>n</i> =73)						
Follicles	29	All cycling and reproductive			10.36 $\pm$ 0.28 ^a (<i>n</i> =73)	0.55 $\pm$ 0.11 ^b (<i>n</i> =73)
Embryos	29					
Parthenotes	15					
					^a (<i>t</i> =3.16, <i>P</i> =0.002)	^b (<i>t</i> =3.94, <i>P</i> <0.0001)

Female reproductive status is represented as: NC, non-cycling; CNR, cycling but non-reproductive; CR, cycling and reproductive. Values with different superscripts differ significantly; $P<0.05$.

cycling animals, there is no significant difference in the timing of ovulation and embryonic development (Fig. 1). We have collected embryos from two induced and three naturally cycling females that appeared to develop faster than normal, probably having the shorter gestation period of 9.5 days shown by Selwood & Woolley (1991).

Parthenote development

Following induction, parthenotes were collected from the uteri of 15 non-mated females ($n=15$) during the first 3 days of gestation and the rate of parthenogenetic development compared with the samples retrieved from naturally cycling animals ($n=15$). In general, parthenotes appeared to develop faster following induction. During the first 24-h post-ovulation (day 1 of gestation), the uteri of naturally cycling animals yielded only unfertilized oocytes, whereas 2- and 4-cell parthenotes were collected from induced animals. On day 2 of gestation, naturally cycling animals yielded parthenotes with a maximum of four cells, compared with 8-cell parthenotes found in the uteri of induced animals. By day 3 of gestation, both the induced and naturally cycling animals contained 8-cell stage parthenotes (maximum cell number), and no further parthenogenetic development was observed.

Pouch young

An additional nine animals (one cycling and reproductive, five cycling but non-reproductive, and three non-cycling) were induced for the birth of offspring (PY). Following induction, three females mated successfully (determined by the presence of sperm in urine samples) and two of these gave birth to viable young. A litter of six PY was born to a previously non-cycling female at 16.7 days post initial injection, corresponding to a gestation period of 10–10.3 days. Another female, cycling and reproductive, gave birth to a litter of six PY at 17.7 days post first injection, corresponding to a gestation period of 11–11.3 days. PY from both litters survived, grew to normal adult size and weight, and were fertile in the following breeding season. The male offspring mated successfully, and the female offspring gave birth to viable young, producing four or five PY per litter.

Discussion

In this study, all non-cycling females bar one responded to the induction protocol and began to cycle. All non-cycling females, examined after day 6 post injection, had ovulated. This highlights the success of the current protocol in recruiting previously non-cycling females to cycle and ovulate. In addition, all cycling but

non-reproductive females responded to the hormone regime, with only one failing to ovulate.

Following induction, fertilized tubal oocytes were detected in oviducts 7.0 days after the first injection, coinciding with the end of the 7-day follicular phase and the day of ovulation in naturally cycling animals. In this study, induced animals ovulated early in the morning, consistent with the findings of Menkhurst *et al.* (2007).

Prior to ovulation, the developing oocytes of advanced antral follicles must resume meiosis and undergo a series of maturational events essential for fertilization (Merry *et al.* 1995). Our findings indicate that the transition of immature germinal vesicle stage oocytes to mature metaphase II stage oocytes may be an extremely rapid process, with meiotic maturation, ovulation, and oviducal fertilization all occurring within a period of <8 h.

Embryonic development of conceptuses obtained following induced ovulation appears to mimic the timing of embryonic development in naturally cycling animals. From time 0, minimum collection times were 7.0 days for tubal oocytes and 7.2 days for uterine zygotes, making oviducal transit possibly as short as 4.8 h. Length of the gestational period following induction, 10–11 days, was within the range expected for this species (Selwood & Woolley 1991). Embryos from a small number of animals appeared to be developing faster than normal, however, this is not unusual as the gestation period can be as short as 9.5 days (Selwood & Woolley 1991) in naturally cycling animals. This may be due to a faster embryonic development at the end of the breeding season, perhaps a mechanism to maximize reproductive success.

Induced ovulation is a routine laboratory procedure in mice, pioneered by Runner & Gates (1954), and has been successfully applied to other small laboratory species such as rats (Goh *et al.* 1992, Jiang *et al.* 1999) and rabbits (Treloar *et al.* 1997), as well as larger domestic eutherians such as cow (Donaldson & Ward 1986, Lopes da Costa *et al.* 2001), horse (Niswender *et al.* 2003), sheep (Leoni *et al.* 2001), pig (Amirov *et al.* 1998), and goat (Kiessling *et al.* 1986). In mice, induced ovulation is used not only for the production of large numbers of oocytes, but also for the establishment of timed pregnancies that proceed to term with the birth of young (Edwards & Gates 1959, Edwards & Fowler 1960, Beaumont & Smith 1975, Spindle & Goldstein 1975). Despite this, protocols in other mammals have focussed largely on the ability to obtain greater numbers of oocytes or embryos for research purposes and often ignored its potential application for conservation purposes.

Induced ovulation may be used to increase the productivity of captive populations, enhancing the reproductive potential of aged, non-cycling or previously non-reproductive females and regulating the timing of mating, ovulation, and fertilization. The protocol presented here, in which hormone dosages

are calculated according to body weight, should be easily adapted for application in related dasyurid species. Within Australia, 17/64 extant dasyurid taxa are threatened (Maxwell *et al.* 1996); however, only one species, *Parantechinus apicalis*, has been systematically bred in captivity for release (Moro 2002). By establishing an induced ovulation protocol effective in the production of live and fertile litters, we are one step closer to aiding the recovery of endangered species through captive breeding coupled with reintroduction or translocation. In the present study, animals responded to induction regardless of age, making this protocol an even more effective conservation tool as age is unknown in many zoo animals collected from the wild.

We present here for the first time, an induced ovulation protocol that is 90% effective in inducing both the cycling and non-cycling females to ovulate, with reduced occurrence of follicle atresia or luteinization, results in normal embryonic development provided that mating occurred successfully and ultimately the production of fertile young. The viability of embryos resulting from induced ovulation was confirmed by taking pregnancies to full term with a fully established time-table of follicular and embryonic development. This time-table can be utilized to enable the collection of precisely timed research material, increasing productivity and reducing loss. The successful production of viable, fertile young, following induced ovulation has enormous potential in increasing the productivity of captive marsupial colonies and assisting the conservation of endangered species through enhanced breeding programs.

Materials and Methods

Animals

The stripe-faced dunnarts used in this study were from a laboratory colony maintained at the University of Melbourne, Department of Zoology, following Australian National Health and Medical Research Council Guidelines for the Care and Use of Animals for Scientific Purposes and held under permits issued by the Department of Sustainability and Environment.

Reproductive monitoring

The reproductive status of all the females was determined by daily monitoring of weight, cells in urine samples, and pouch changes throughout the breeding season (Hickford *et al.* 2001). The follicular phase of the estrous period is associated with a transient increase then fall in weight over 7 days, and the presence of cornified epithelial cells in the urine. If conceptuses are required, a male is introduced when cornified epithelial cells peak, usually between days 4 and 6 of the follicular phase, and mating is detected by the presence of spermatozoa in the urine. Spontaneous ovulation (time 0 of gestation) occurs at the end of the follicular phase and is associated with a fall in weight and the appearance of many

polymorphonuclear leucocytes in the urine. The following day, marked by an increase in weight, is the first day of gestation. The luteal phase is contained within the gestation phase and is maintained until day 9 of gestation. *S. macroura* has a mean gestational period of 10.7 days (Selwood & Woolley 1991). The end of gestation is indicated by a sharp drop in weight, accompanied by yellow crystals and sometimes red blood cells in the urine, a clear secretion in the pouch and PY if pregnant (Selwood & Cui 2006). The period between the end of these changes in a non-pregnant cycle and the beginning of the next follicular phase is termed the intermediate phase.

Treatments

The majority of animals employed in this study were in their first breeding season; 85% (62/73) of control (naturally cycling) and 84% (48/57) of experimental (induced to ovulate). The remaining animals were used during their second breeding season. Females were grouped according to their reproductive status: cycling and reproductive, cycling but non-reproductive, and non-cycling. Cycling females that had either failed to mate or had mated but produced no PY over 1–8 months of reproductive monitoring were categorized as cycling but non-reproductive. In this study, all control animals were cycling and reproductive, whereas many animals selected for induction showed poor reproductive performance. Cycling females, whether reproductive or non-reproductive, were induced during the follicular, late luteal (day 6–10 following ovulation), or intermediate phase of the estrous cycle, as treatment during early-mid luteal phase has proven less successful (Menkhorst *et al.* 2007). Females in which the estrous cycle could not be determined by daily monitoring (non-cycling females) were induced to stimulate their first cycle and/or regulate their estrous cycle profile. Ovulation was induced using a series of two hormone injections delivered in calcium- and magnesium-free PBS. Menkhorst *et al.* (2007) found no difference in the time of ovulation after morning or evening stimulation, so to make hormone administration more convenient, we gave both the injections at 1600 h and also returned to the use of body weight to calculate the appropriate hormone dosage, giving each female a total of 0.1 IU eSG/g. At time 0, animals received a dose of 0.06 IU eSG/g (Folligon; Allhank Trading Co., South Melbourne, Victoria, Australia), followed on day 4 by a second injection of 0.04 IU eSG/g (Hickford *et al.* 2001). The volume required per injection was determined for individual females on the day of induction, and hand-warmed prior to i.p. injection.

Analysis

Females were killed by inhalation of Halothane (Rhone Merieux, West Footscray, Victoria, Australia), followed by cervical dislocation. The reproductive tracts were removed, washed in warmed PBS[−] (35 °C) and examined under a dissecting microscope (Zeiss, North Ryde, New South Wales, Australia) to confirm estrous cycle stage. An inverted microscope (Wild Leitz, Melbourne, Victoria, Australia) with heated stage was employed for closer examination of ovarian follicles, oocytes, zygotes, conceptuses, and parthenotes.

To examine follicle development, ovaries were collected on the last 3 days of the follicular phase of the estrous cycle. Mean follicle diameters were calculated by measuring the longest axis, and the axis perpendicular, using a calibrated ocular micrometer. Follicles were determined to be primary, secondary, tertiary, or antral based on size and morphology (Kress *et al.* 2001, Nation & Selwood 2009). Antral follicles were carefully ruptured using 29 G needles to assess oocyte maturity (Merry *et al.* 1995). As ovulation occurs at 0700 h (Menkhorst *et al.* 2007), females were killed late on the day of ovulation and on subsequent days (at closer intervals during days 1–3 of gestation) to collect stage-specific conceptuses. Parthenotes were collected from non-mated females during the first 3 days of the gestational period to confirm ovulation and to examine the rate of parthenogenetic development. Ovarian corpora lutea were counted to determine ovulation number and corpora albicans to establish previous cycling activity. Oviducts were examined under a transmitted light microscope for the presence of oocytes. Each uterus was transferred to warmed culture medium (DMEM; Sigma–Aldrich), slit longitudinally along the midline and carefully inverted so embryos or parthenotes, if present, could roll out gently into the medium. Embryos were examined for evidence of fertilization, and assessed for developmental stage and normality (Selwood & McCallum 1987). PY born following induced ovulation were raised in the colony and their fertility was assessed during the following breeding season by incorporating them into the normal breeding program.

Statistical analysis

Independent sample *t*-tests were employed to examine the effect of animal age on the ability of females to begin cycling and/or ovulate following induction. The number of developing antral follicles, corpora lutea and atretic follicles per ovary was calculated as the mean ($\pm$ S.E.M.) and compared between induced and naturally cycling groups by independent sample *t*-tests. Values were considered statistically significant when $P < 0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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