Comparison of the production, quality, and *in vitro* maturation capacity of oocytes from untreated cycling and intermediate phase equine serum gonadotropin-treated fat-tailed dunnarts (*Sminthopsis crassicaudata*)

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Abstract

This study describes ovarian changes during the natural and stimulated reproductive cycle of breeding (≤ 12 month) and retired (> 12 month) fat-tailed dunnarts, *Sminthopsis crassicaudata*. Increased urinary cornified epithelial cells and the influx of leukocytes defined day 0, at which time the naturally cycling females had already ovulated; at day 16 females had no antral follicles, but by day 20 antral follicles had begun to develop. There was no difference between naturally cycling breeding and retired females. Females were stimulated with 1 IU equine serum gonadotropin (eSG) during the intermediate phase on day 16 and killed 3, 4, or 5 days later. Stimulation resulted in a significant increase in the number of growing antral follicles but retired females demonstrated a reduced response. Upon collection from breeding females 4 days following eSG stimulation, 100% of oocytes were at the first polar body (PB1) stage, those collected from retired females were immature upon collection but within 48 h $98.2 \pm 1.9\%$ were cultured to the PB1 stage. The rate of ovulation was high in breeding females 5 days following stimulation but retired females were less reliable, and in both groups all oocytes were degraded. This is the first study to describe a reliable technique, involving ovarian stimulation during the intermediate phase and segregation of age groups, allowing the collection of a large number of healthy PB1 stage oocytes from *S. crassicaudata*. This is important for the development of further assisted reproductive techniques for this species and threatened dasyurids.

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Introduction

The fat-tailed dunnart, *Sminthopsis crassicaudata*, is a small (10–20 g), short-lived and generally solitary dasyurid marsupial found in central and southern Australia (Morton 1995). Sexual maturity in females is reached at 3 months of age and in captivity photoperiod manipulation allows continuous breeding until females reach 30 months of age (Smith *et al.* 1978, Bennett *et al.* 1990). The reproductive cycle lasts 31 days and in this polyovular species ~ 14 oocytes are ovulated, although up to 24 embryos have been observed (Smith & Godfrey 1970, Bennett *et al.* 1990). After a gestation period of 13 days, females give birth to supernumerary young, as only eight to ten teats are present (Godfrey & Crowcroft 1971, Bennett *et al.* 1990). The young who successfully attach to a teat are weaned at ~ 70 days (Godfrey & Crowcroft 1971).

The relative ease of housing, breeding, and handling of *S. crassicaudata* makes them good experimental models for the study of assisted reproductive techniques (ART) for application to larger threatened dasyurids such

as the Tasmanian devil, *Sarcophilus harrisii* (6–8 kg), and northern quoll, *Dasyurus hallucatus* (350–1200 g). There is an increasing role for the use of ART in marsupial conservation but difficulties arise in species where our understanding of reproductive physiology is limited. A key issue for the implementation of ART is the development of protocols to stimulate or synchronize female reproductive activity (Rodger *et al.* 2009).

The first study to report ovarian stimulation protocols in *S. crassicaudata* examined adults (> 4 months of age) and used 20 IU equine serum gonadotropin (eSG), resulting in ovulation and mating but not live births (Smith & Godfrey 1970). Subsequent studies examined females at random points in their reproductive cycle and found that 3- to 5-month-old females treated with 1 or 5 IU eSG ovulated within 5–6 days (Rodger *et al.* 1992). However, results were variable as only a proportion of females ovulated and at the higher dose overstimulation was recorded (Rodger *et al.* 1992). Additional investigations indicated that doses as low as 1.5 IU caused overstimulation and luteinization of follicles in animals

3–6 months of age (M Smola & J C Rodger, unpublished observations). Although *S. crassicaudata* embryos have been produced (Breed & Leigh 1996) and a single litter has been born (Rodger *et al.* 1992) following the use of these protocols, a proportion of stimulations failed. Although reduced reliability is acceptable for morphological studies (Anderson & Breed 1993, Breed & Leigh 1996), it is less appropriate for the development of ART.

An explanation for the variable results may be the presence or absence of active corpora lutea (CL). In marsupials, the CL persists through the majority of the luteal phase and does not regress when the female is treated with exogenous hormones (Tyndale-Biscoe *et al.* 1974). To avoid these CL effects, ovarian stimulation has been carried out in juvenile females (<18 weeks of age; Smith & Godfrey 1970). Alternatively, reproductive monitoring can be carried out to avoid the luteal phase and studies in the stripe faced dunnart, *Sminthopsis macroura*, demonstrate superior results when females are stimulated during the intermediate or follicular phase (Hickford *et al.* 2001, Menkhorst *et al.* 2007). Reproductive monitoring involves examining the presence of urinary cornified epithelial cells (CEC) that are an indicator of elevated estradiol (E_2) concentrations and thus the late follicular phase (de Brux 1958). CEC can remain elevated for several days prior to an influx of leukocytes (Woolley 1990) and this period of time will be defined as the 'cytological estrus' for the purpose of this study. Monitoring CEC in *S. crassicaudata* has demonstrated that estrus and mating occurs at times of increased CEC and that early zygotes are found during periods of high CEC (Godfrey 1969, Godfrey & Crowcroft 1971, Bennett *et al.* 1979, Selwood 1987). However, neither the precise timing of ovulation nor its relationship to elevated CEC is known.

For studies examining oocyte-based ART for marsupials, the collection of ovulated oocytes from the reproductive tract is not an option due to the secretion of the oviductal mucoid and shell coat, which surrounds the oocyte and acts as a block to sperm penetration (Rodger 1990). Alternatively, oocytes from pre-ovulatory ovarian antral follicles may be collected and reach nuclear maturation *in vitro*. In naturally cycling eutherians and marsupials, *in vitro* maturation of oocytes from preovulatory antral follicles is spontaneous and requires

Table 1 Reproductive attributes of naturally cycling *Sminthopsis crassicaudata* at day 0 ($n=13$), determined by the presence of leukocytes following a period of high cornified epithelial cells in urine samples, day 16 ($n=4$), and day 20 ($n=9$).

	Uterine size (mm ²)	Corpora lutea diameter (μm)	Antral follicles	Ovulation
Day 0	51.57 ± 9.07 ^a	396.20 ± 12.55 ^a	No	Yes
Day 16	13.27 ± 0.68 ^b	525.28 ± 14.62 ^b	No	No
Day 20	8.69 ± 0.86 ^b	397.55 ± 8.73 ^a	Yes	No

Values with different letters are significantly different (P at least <0.05).

no exogenous hormonal support (Pincus & Enzmann 1935, Selwood & VandeBerg 1992). Spontaneous maturation also occurs in most marsupials following stimulation with eSG, but not FSH (Mate & Rodger 1993, Mate & Buist 1999, Glazier *et al.* 2002). However, in *S. macroura* eSG, stimulated oocytes collected from antral follicles do not reliably reach nuclear maturation (Merry *et al.* 1995) and the addition of LH to culture media only resulted in maturation rates of 60% (Maleszewski & Selwood 2004).

This study aimed to develop high-yield protocols for the collection of first polar body (PB1) stage oocytes from two age classes (≤ 12 months and > 12 months) of *S. crassicaudata*. To achieve this, we used urinary CEC and the influx of leukocytes to examine ovarian activity, oocyte maturation and oocyte quality in the unstimulated cycle. Subsequently, we stimulated females with eSG during the intermediate phase, as defined by CEC and leukocytes. The development of the reproductive tract, ovary and nuclear status of oocytes was assessed 3, 4, and 5 days later. In addition, oocytes were placed in culture without exogenous hormone supplementation and their nuclear development was assessed up to 48 h later.

Results

Cytological estrus was identified in 72 (84%) out of the 86 animals monitored. General health issues or pouch tumors (benign fibroadenoma or malignant adenocarcinoma) caused ten animals to be removed from the experiment and 14 animals were unable to be monitored as they regularly defecated upon handling or would not urinate.

Figure 1 *Sminthopsis crassicaudata* granulosa cell-oocyte complex (GOC) from a preantral follicle collected at day 16 with tightly bound granulosa cells (A) or a day 20 GOC (B) where the granulosa cells are able to be removed to reveal a germinal vesicle stage oocyte following staining with Hoechst 33342 (C); bar = 40 μm.

Experiment 1: timetable of reproductive events in natural cycles

In natural cycles, females assessed at D0 had recently ovulated and their uterine tissues were large, turgid, and well vascularized (Table 1). The number of oocytes ovulated in the breeding (10.60 ± 1.16 , $n=5$) and retired (11.75 ± 1.94 , $n=8$) females did not vary. At D16, uterine tissues were less vascularized and significantly smaller ($P < 0.05$, Table 1) and the ovary was dominated by large CL (Table 1). No antral follicles were observed and oocytes collected from preantral follicles were tightly surrounded by granulosa cells that could not be removed by manual pipetting (Fig. 1A). By D20, the CL had an irregular, creased surface and were significantly smaller ($P < 0.001$, Table 1). A small number of antral follicles were observed and the number of antral follicles observed in breeding (4.75 ± 0.59 , $n=4$) and retired (3.4 ± 0.08 , $n=5$) females did not vary; hence the data were combined for subsequent analysis. Oocytes extruded from antral follicles were surrounded by granulosa cells that when removed revealed germinal vesicle (GV) stage oocytes ($n=32$, Fig. 1B and C).

Effect of the length of cytological estrus on ovulated oocyte quality

Females with a 2-day cytological estrus had oocytes with a visible yolk mass, two polar bodies, and female pronuclei ($n=24$; Fig. 2A and B). Cleavage was rare but if present it represented parthenogenetic activation with both cells indicating morphologically normal nuclear material. Females who had a 3-day cytological estrus were mostly pre-cleavage or parthenogenetic (Fig. 2C and D) but 15% ($n=26$) showed fragmentation and contained segments lacking nuclear material. In females who had a cytological estrus that lasted 4 or more days, 45% of oocytes were fragmented ($n=36$; Fig. 2E and F).

The length of cytological estrus was determined in 52 (69%) out of the 75 cycles where estrus was determined due to the alternate day sampling regime ($n=72$, three animals cycled twice). There was no discernable pattern in the proportion of females which had a cytological estrus for 2, 3, or 4 or more days although the latter was the most common occurring in 22 (42%) out of the 52 defined cycles.

Experiment 2: stimulation of females with eSG

Stimulation significantly increased the number of antral follicles in both breeding and retired females at D16+3 and D16+4 when compared with unstimulated D20 females, but retired females had significantly fewer antral follicles than breeding females (Fig. 3).

Figure 2 Morphology of naturally ovulated oocytes from *Smynthopsis crassicaudata* collected after a cytological estrus which lasted: 2 days showing polar body extrusion and a pronuclei (A and B), 3 days showing parthenogenesis with nuclear material in each cell (C and D), or 4 or more days showing fragmentation (E and F) seen with light microscopy (A, C and E) or following staining with Hoechst 33342 (B, D and F); bar = 40 μ m.

Stimulation significantly increased the size of antral follicles in both breeding and retired females. Antral follicles from D16+3 and D16+4 were larger than those from unstimulated D20 females and D16+4 antral follicles were significantly larger than those from D16+3 breeding and retired females but the D16+4 antral follicles from retired females were smaller than those from younger breeding females (Fig. 4). No ovulation was observed in D16+3 or D16+4 breeding or retired females.

Figure 3 The number of antral follicles in *Smynthopsis crassicaudata*. Data are combined at day 20 (D20) of an unstimulated cycle and presented separately from retired females (> 12 months of age, filled bars) and breeding females (≤ 12 months of age, unfilled bars) either 3 (D16+3) or 4 (D16+4) days following stimulation with 1 IU equine serum gonadotropin on day 16 of their cycle. Values with different letters are significantly different (P at least < 0.05).

Figure 4 The diameter of antral follicles in *Sminthopsis crassicaudata*. Data are combined at day 20 (D20) of an unstimulated cycle and presented separately from retired females (> 12 months of age, filled bars) and breeding females (≤ 12 months of age, unfilled bars) either 3 (D16+3) or 4 (D16+4) days following stimulation with 1 IU equine serum gonadotropin on day 16 of their cycle. Values with different letters are significantly different (P at least < 0.01).

In vitro maturation

Upon collection from breeding females 3 days following eSG stimulation, oocytes were GV or germinal vesicle breakdown (GVBD; $n=23$) and after 48 h in culture only $20.83 \pm 20.83\%$ demonstrated nuclear maturation to the PB1 stage ($n=18$). Prior to culturing only $17.50 \pm 9.38\%$ of oocytes from retired females had undergone GVBD ($n=22$) and after 48 h $38.63 \pm 8.61\%$ had reached the PB1 stage, with none remaining at the GV stage ($n=26$).

Oocytes harvested from breeding females at D16+4 were all at the PB1 stage ($n=33$) upon collection; they appeared normal with no evidence of fragmentation (Fig. 5A and B). In retired females collected at D16+4, $77.06 \pm 8.68\%$ of oocytes had undergone GVBD but only $6.67 \pm 6.67\%$ had reached the PB1 stage ($n=34$). Following *in vitro* culture for 24 h, the proportion of PB1

oocytes significantly increased to $62.06 \pm 14.02\%$ ($P < 0.01$, $n=34$) and by 48 h $98.18 \pm 1.92\%$ of oocytes had achieved nuclear maturation to the PB1 stage ($n=26$). There was no evidence of fragmentation of *in vitro* matured oocytes (Fig. 5C and D).

Induced ovulation

Ovulation was observed in five out of six breeding D16+5 females but oocytes were low quality, demonstrating evidence of cytoplasmic breakdown, blebbing, and non-specific cytoplasmic staining (Fig. 6A–C). Only two out of six retired females had ovulated by D16+5, and oocytes were again degraded. Hormone administration was confirmed in non-ovulating retired females by the presence of highly vascularized uterine tissues that were significantly larger than that of D0 females ($84.26 \pm 9.56 \text{ mm}^2$, $n=4$, $P < 0.05$). The rate of ovulation in breeding (17.00 ± 2.17 , $n=5$) and retired females (17.5 ± 0.50 , $n=2$) did not vary. However, both were significantly greater than the natural rate of ovulation for retired ($P < 0.01$) and breeding females ($P < 0.05$) as presented in experiment 1.

During this study, there was no evidence of over-stimulation, typified by opaque follicles still containing oocytes, or negative effects of the stimulation protocol on *S. crassicaudata* with regular activity, grooming, and socialization maintained. However, increased aggression toward researchers typified by vocalizations and biting was observed following eSG treatment as is seen during the normal cytological estrous period.

Discussion

The results from this study provide the first detailed description of follicular development at several points during the estrous cycle in unstimulated and eSG stimulated *S. crassicaudata*. We demonstrate that a significantly larger cohort of oocytes can be harvested from antral follicles following stimulation with 1 IU eSG and that if collected 4, but not 3, days post stimulation they reliably undergo nuclear maturation to the PB1 stage without additional hormone supplementation. We have also demonstrated that females that are older than 12 months of age have a reduced response to eSG stimulation, but this difference is not apparent in non-stimulated cycles.

CEC monitoring

The period of increased CEC, dictated by elevated E_2 concentrations, which results in proliferation and desquamation of vaginal epithelial cells, was used in the present study to designate cytological estrus (de Brux 1958, Regli & Kress 2002). The use of monitoring CEC in *S. crassicaudata* has been broadly described by

Figure 5 First polar body stage oocytes from breeding age (≤ 12 months of age) *Sminthopsis crassicaudata* upon collection (A and B) or a retired female (> 12 months of age) following 24 h *in vitro* culture (C and D) after stimulation with 1 IU equine serum gonadotropin on D16 of their cycle seen with light microscopy (A and C) or following staining with Hoechst 33342 (B and D); bar = 40 µm.

Figure 6 Degraded ovulated oocytes from *Smynthopsis crassicaudata* stimulated with 1 IU equine serum gonadotropin on D16 of their cycle and collected from the uterus 5 days later viewed with a light microscope (A) or following staining with Hoechst 33342 (B and C); bar = 50 μ m.

Godfrey (1969), Godfrey & Crowcroft (1971) and Bennett *et al.* (1979) who reported estrus and mating at times of increased CEC. But the present study was the first to specifically examine timing of ovulation in *S. crassicaudata*.

Our findings demonstrated that the presence of leukocytes in urine samples did not indicate the day of ovulation as seen in *S. macroura* (Selwood & Woolley 1991). Nor did ovulation coincide with a decrease in CEC as observed in another dasyurid, *Antechinus stuartii* (Selwood 1980). Instead, the increasing degradation of ovulated oocytes from females with an increasingly long cytological estrus indicated that ovulation occurs when CEC are initially elevated. This concept is supported by the observations of Smith & Godfrey (1970) who describe the onset of estrus marked by increasing CEC and ovulation occurring soon after this point in *S. crassicaudata*. Furthermore, Selwood (1987) described *S. crassicaudata* having early zygotes during periods of high CEC and 4–16 cell embryos following the decline of CEC. Finally, preliminary investigations into specifically timed *S. crassicaudata* breeding trials have indicated that pairs mate on the first day of score 3 CEC but not once leukocytes are observed (data not shown). Hence, we suggest that the influx of leukocytes is indicative of the end of the estrous period. Although not indicative of ovulation, in this study the presence of leukocytes was maintained as D0 as it allows allocation of a defined time point without retrospective assessment. The underestimation in timing of events caused by the nominated D0 falling 1–4 days after ovulation means that females may be further into the intermediate phase than initially assumed. However, the potential variability in endocrine status was not reflected in the response to eSG treatment, which was highly consistent.

Parthenogenesis and fragmentation in naturally ovulated oocytes

The parthenogenetic activation seen in oocytes from females with a cytological estrus of 3 days was not unexpected, nor is the degradation and fragmentation which occurs after a 4-day cytological estrus. Parthenogenesis is reported in 35% of uterine oocytes collected from eSG stimulated *S. crassicaudata* and fragmentation is reported for those that have undergone

more than two divisions (Anderson & Breed 1993). This has been suggested to relate to inbreeding within the *S. crassicaudata* captive colony (Anderson & Breed 1993) and the increased rates of parthenogenetic activation observed in the present study may represent further inbreeding pressure within the *S. crassicaudata* colony 15 years on. Nonetheless, the colony's ongoing ability to breed naturally indicates that oocytes are still capable of fertilization and production of live young.

Timetable of events in natural cycles

Given that the reproductive cycle of *S. crassicaudata* lasts 31 days with a gestational period of 13.5 days (Godfrey & Crowcroft 1971, Bennett *et al.* 1990), we suggest that if D0 is nominated as the end of estrus then D16 females will be within the intermediate phase. This was observed experimentally with D16 females indicating no follicular development. D16 females also demonstrated the largest CL but it is unlikely that these were still secreting progesterone as they were smaller in size than mid- to late-luteal phase CL from other dasyurids (Woolley 1966, Selwood & Woolley 1991). In addition, progesterone concentrations decline before regression of the CL which can occur at parturition or the end of a non-pregnant cycle (Hinds & Selwood 1990, Woolley 1990, Selwood & Woolley 1991). The smaller size of the CL and the evidence of consistent patterns of dasyurid luteal regression in the literature suggested that the D16 ovary was not governed by progesterone and would be receptive to exogenous stimulating factors as described in studies of *S. macroura* (Hickford *et al.* 2001, Menkhorst *et al.* 2007).

Females examined at D20 were expected to be in the follicular phase and have a young cohort of small hormone-dependent antral follicles. This was demonstrated experimentally in the current study that showed that oocytes were at the GV stage and had not begun nuclear maturation. Furthermore, the CL were rapidly shrinking which has also been previously reported in other dasyurids (Hinds & Selwood 1990, Selwood & Woolley 1991).

The observation that mechanical removal of granulosa cells from the D16 oocytes was not possible, but that their removal from D20 oocytes was, is likely to be due to immature granulosa–oocyte complexes (GOC)

maintaining cellular processes from granulosa cells that form gap and tight junctions through the zona pellucida for endocrine and nutritional support of the oocyte (Breed & Leigh 1990). This was not observed in more mature GOC in preparation of the complete removal of granulosa cells prior to ovulation (Breed & Leigh 1988). Removal of adherent granulosa cells with hyaluronidase was described in *S. macroura* (Merry *et al.* 1995) but was not able to be reliably achieved in the present study (data not shown), assumedly because marsupials do not utilize hyaluronic acids within their surrounding cell layers (Chapman & Breed 2006). Nonetheless, subsequent trials have shown treatment with the protease trypsin to be more successful (2.5 g/ml for 5 min; N A Czarny, unpublished observations).

Stimulation of females with eSG

Superovulation has been used in monovular marsupials such as the Tammar wallaby, *Macropus eugenii*, and brushtail possum, *Trichosurus vulpecula*, to overcome the selection of one dominant follicle (Rodger & Mate 1988, Molinia *et al.* 1998) but these techniques may also be used with polyovular marsupials such as dasyurids to increase, control, and manipulate oocyte maturation (Rodger *et al.* 1992, Merry *et al.* 1995). This study demonstrated that a timed 1 IU eSG stimulation protocol resulted in the recruitment of significantly more follicles than in unstimulated cycles, and that this increase is greater in younger (≤ 12 month of age) breeding females. Oocytes from breeding females harvested 4 days following eSG stimulation demonstrate nuclear maturation and those collected from retired females are reliably cultured to the PB1 stage within 48 h.

In vitro maturation

In vitro maturation is an important step toward the generation of protocols for the collection of gametes for future studies of IVF or ICSI. This is the first report of a reliable protocol for harvesting GVBD oocytes in *S. crassicaudata* and their subsequent culture to the PB1 stage in the absence of endocrine support. The ability of oocytes collected 4 days following stimulation to undergo nuclear maturation to the PB1 stage is not unexpected as maturation of oocytes from antral follicles in natural cycles and eSG stimulated females is spontaneous and requires no additional hormonal support in many species, including marsupials (Pincus & Enzmann 1935, Selwood & VandeBerg 1992, Mate & Rodger 1993, Glazier *et al.* 2002). We also show that the less mature oocytes collected 3 days following stimulation with eSG could not reliably reach the PB1 stage within 48 h in the absence of exogenous hormones, similar to the outcomes in granulosa cell enclosed

oocytes from *S. macroura* (Merry *et al.* 1995). Subsequent experiments are suggested to determine the maturation potential of these follicles following *in vitro* maturation with a longer culture period or the addition of exogenous LH.

Oocytes harvested from breeding females 4 days following stimulation were the most mature, with 100% at the PB1 stage, those harvested from smaller antral follicles of retired females were predominantly GVBD. Although there is a significant size difference between antral follicles in these two datasets, GVBD oocytes were collected from follicles as large as 650 μm and PB1 stage oocytes were collected from follicles as small as 437 μm . This variation in follicle size makes establishing a threshold follicle size for collection of PB1 stage oocytes difficult; however, the production of PB1 stage oocytes can still be readily achieved by the robust *in vitro* maturation protocol or use of breeding age females.

The LH surge and induced ovulation

Although eSG mimics the effect of FSH and LH, some marsupials require an additional drug to induce the resumption of meiosis and ovulation (Rodger & Mate 1988, Jungnickel *et al.* 2000). In *S. crassicaudata*, the eSG stimulation was reported sufficient to generate an endogenous LH surge (Rodger *et al.* 1992). This is supported by the present study as the resumption of meiosis (observed as GVBD) occurs as early as 3 days following stimulation, presumably in response to the LH surge.

LH activity is also indicated by the high rate of ovulation observed in breeding females when examined 5 days following stimulation. However, the response to stimulation was less consistent in retired females where only two out of six females ovulated. Nonetheless, these females may have ovulated given an extra day as their uterine tissues demonstrated increased vascularization, an effect of increased concentrations of LH. Previous studies saw Rodger *et al.* (1992) stimulate adult *S. crassicaudata* with 1 IU eSG during undefined stages of the reproductive cycle and found ovulation occurred in most females within 6, but not 5, days. Variable rates of ovulation were also observed with an untimed 1 IU eSG protocol examining animals that were on average 12 months of age (Anderson & Breed 1993, Breed *et al.* 1994, Breed & Leigh 1996). Finally, similar results were observed in *S. macroura* that were induced in the intermediate phase, where although the timing of ovulation was improved from prior studies, it varied by up to 4 days (Menkhorst *et al.* 2007).

In the females that did ovulate, the oocytes were degraded and blebbing with non-specific nuclear staining. Although eSG has a long half life and can result in overstimulation (Smith & Godfrey 1970, Rodger *et al.* 1992, Hickford *et al.* 2001), this is unlikely

to be the case in the present study as the dose used was up to 20 times lower than that used in previous *Sminthopsis* studies (Smith & Godfrey 1970, Menkhorst *et al.* 2007). We also observed no leutinized follicles or negative effects that were examined on a nine-point animal monitoring scale. Our low dose was however sufficiently high to stimulate the growth of increased numbers of follicles and initiate their maturation. Instead, degraded ovulated oocytes may be the result of a delayed LH surge causing oocytes to be retained in the follicle for longer than necessary (Malhi *et al.* 2006). It would be of interest to increase our understanding of this by examining the endocrine profiles or CEC patterns of stimulated females in subsequent studies as examined following stimulation in *M. eugenii* (Jungnickel & Hinds 2000).

Improvement to the protocol

The present study describes increased consistency following stimulation with eSG at D16 compared with previous untimed studies (Smith & Godfrey 1970, Rodger *et al.* 1992, Anderson & Breed 1993, Breed & Leigh 1996). Furthermore, we found age-based differences in the response to stimulation which may also have contributed to the variation observed in previous studies. This age-based difference in ovarian activity was not observed in natural cycles but following stimulation older females had fewer and smaller antral follicles and their oocytes were less mature than those of younger females. Our results are not surprising as *S. crassicaudata* is a short-lived species with a limited reproductive life and a reduced response to ovarian stimulation with increased age is also observed in other species. Older cows have a reduced number of recruited follicles after superovulation (Malhi *et al.* 2006) and older women have a reduced response to exogenous stimulatory hormones (Jacobs *et al.* 1990, Dew *et al.* 1998).

This age factor is an important finding that unexpectedly reveals an easily avoidable source of variation observed in previous studies examining ovarian stimulation in *S. crassicaudata*. Our results have the additional benefit of describing the use of retired animals for experimental applications, thus developing a tool for more efficient use of oocyte donors and improved colony management.

Conclusion

This study has resolved two major sources of variation in ovarian stimulation protocols. We have increased the reliability of the response to stimulation by treatment during the intermediate phase and we describe a reduced response to stimulation in older females. An understanding of these aspects has led to the

development of a high-yield protocol that enables the collection of oocytes able to be cultured to the PB1 stage without exogenous hormones. Oocytes demonstrating nuclear maturation can be used in future studies of ART in dasyurid marsupials including the molecular and physical aspects of fertilization, stimulation of non-seasonal breeding, and synchronization of estrous cycles for subsequent embryo transfer.

Materials and Methods

Husbandry

S. crassicaudata were sourced from the Marsupial Facility at the University of Newcastle and originated from the long-established colony at the University of Adelaide. Females had no access to males and were housed in groups of up to four in opaque polypropylene boxes (420×280×160 mm) with a recycled paper substrate, shelters, and paper to build nests. They had *ad libitum* access to food (IAMS chicken adult cat food, Dayton, OH, USA) and water. The animals were exposed to a 16 h light:8 h darkness cycle to promote constant breeding (Smith *et al.* 1978, Bennett *et al.* 1990). This study used breeding age females (≤ 12 months of age) and those who had been retired from the colony's breeding population (> 12 months of age). Retired females were still capable of natural breeding and represent a readily available pool of oocyte donors for experimental studies while not compromising the sustainability of the breeding colony.

The use of protected native species was licensed by New South Wales National Parks and Wildlife Service (Australia) and all experiments were approved by Newcastle University Animal Care and Ethics Committee.

Reproductive state was assessed every second day by collection of urine onto glass slides ($n=86$). Samples were examined on a microscope at $\times 250$ and the proportion of CEC was assessed across no fewer than five fields of view. A three-point scoring system was used to assess the presence of CEC (score 1: one to two cells per field of view, score 2: three to eight cells per field of view, and score 3: more than eight cells per field of view). When samples containing score 2 CEC animals were checked daily until they reached score 3 followed by an influx of leukocytes – this day was designated as day 0 (D0). The success of CEC monitoring was determined by the proportion of monitored females for which estrus was correctly nominated and reasons for exclusion from the protocol, such as reluctance to urinate or illness, were recorded.

Gross morphology

Females were killed between 0800 and 1100 h by CO₂ inhalation and the reproductive tract was transferred into warm (35 °C) PBS (Invitrogen) under a dissection microscope. The length and width of the uterus was measured with an eyepiece graticule and the uterine area was calculated as an index of uterine size using the formula for the area of an oval

($\pi \times \text{half the width} \times \text{half the length}$). Ovaries were removed, washed in warm PBS, and transferred to bench media that were comprised of HEPES buffered DMEM (pH 7.4; Sigma–Aldrich) supplemented with 10% (v/v) FCS (Trace Biosciences, Castle Hill, NSW, Australia), 100 IU/ml penicillin, and 100 µg/ml streptomycin (Invitrogen).

Follicular morphology

The diameters of CL and follicles were measured with an eyepiece graticule CL were measured within the ovary but individual follicles were isolated by dissection, using a 27.5 gage needle mounted on a 0.5 ml syringe (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Follicles were classed as preantral if no space between the oocyte and the follicle was observed and antral when a crescent-shaped space was seen.

Oocyte collection and staining

Syringe-mounted needles were used to flush ovulated oocytes from the uterus or extrude GOC from the isolated follicles. Oocytes were either used for culture without further treatment (see below) or stripped of granulosa cells using a glass pipette and stained to determine the state of maturation. The degree of granulosa cell adhesion was recorded then oocytes were washed in PBS, stained for 20 min in 10 µg/ml Hoechst (H33342, Sigma–Aldrich) and then washed thrice in PBS (Mate & Buist 1999). To aid in visualization, the oocytes were mounted on slides with the coverslip supported by a 9:1 mixture of vaseline and candle wax.

Nuclear maturation was assessed by viewing on an inverted microscope (Zeiss, Jena, Germany) at $\times 200$ and $\times 400$ using filter set 2 (exciter filter 365 nm, emission filter 420 nm). Oocytes were classed as GV, GVBD, PB1, parthenogenetic, or fragmented.

Experiment 1: timetable of reproductive events in natural cycles

To determine the best time for stimulation with eSG, three time points in the reproductive cycle were examined – day 0 (D0) represented the estrous period, day 16 (D16) represented the intermediate phase, and day 20 (D20) represented the follicular phase. At D0, 16 and 20 retired females (> 12 months of age) were assessed as described above. Breeding females (≤ 12 month of age) were also assessed at D0 and D20 to identify the presence of age-related differences in the number of oocytes ovulated and antral follicles. In all females, the size and contents of the uterus, size and state of the ovaries including CL and follicles, the adherence of granulosa cells, and nuclear maturation of oocytes were examined.

Initial experiments indicated high variation in the oocyte quality of D0 females, which appeared to be related to the period of time that elevated CEC were observed prior to the influx of leukocytes – defined as the length of the cytological estrus. To further examine this aspect, oocyte quality was compared in D0 females which were grouped according to the length of their cytological estrus being 2 ($n=3$), 3 ($n=3$) or 4,

or more ($n=3$) days. To determine the frequency of occurrence, the length of cytological estrus was assessed for each of the 75 cycles where estrus was determined.

Experiment 2: stimulation of females with eSG

Females were given 1 IU eSG (Folligon, Intervet, Boxmeer, Holland) i.p. while anesthetized with 4% isoflurane (Virbac Animal Health, Peakhurst, NSW, Australia) between 0800 and 1100 h on D16 of their cycle. The welfare of stimulated females was examined daily with a nine-point monitoring scale, which assessed body condition and behavioral aspects. Females were killed 3 (D16+3), 4 (D16+4), or 5 (D16+5) days following stimulation and processed as described above. Each group contained at least four individuals and breeding and retired females were assessed separately.

Oocyte maturation without exogenous hormone supplementation was carried out on GOC from breeding ($n \geq 4$) and retired ($n \geq 3$) D16+3 and retired D16+4 ($n \geq 5$) females. GOC were pipetted by mouth from bench media into sterile culture media containing DMEM (pH 7.4; Sigma–Aldrich) supplemented with 10% (v/v) FCS and 100 IU/ml penicillin and 100 µg/ml streptomycin (Selwood 1987, Merry *et al.* 1995). Nuclear maturation of the stripped oocytes was assessed upon collection and for up to 48 h using H33342 as described above.

Statistical analysis

Mean values were presented $\pm$ S.E.M. and significant differences were determined using *t*-tests in Microsoft Excel or ANOVA followed by *post hoc* Tukey's tests in JMP (SAS Institute Inc., Cary, NC, USA) for parametric data. Oocyte maturation data did not follow a normal distribution and was assessed with the non-parametric Kruskal–Wallis one-way ANOVA in JMP.

Declaration of interest

The authors declare there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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