REPRODUCTION
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Semen-induced luteal phase and identification of a LH surge in the koala (*Phascolarctos cinereus*)

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Abstract

The koala ovulates in response to mating. The purpose of this study was to document the LH surge induced by copulation and to investigate the potential roles of mechanical stimulation of the urogenital sinus and deposition of semen in induction of the luteal phase. In experiment 1, serial blood samples from four koalas that underwent normal mating showed elevated concentrations of LH approximately 24–32 h post-coitus. There was no corresponding elevation in LH in koalas ($n = 4$) that were exposed to the presence of a male but received no physical contact. In experiment 2, koalas on day 2 of oestrus were exposed to one of the following treatments ($n = 9$ per group): artificial insemination with 1 ml 0.9% sterile saline (control group), insemination with 1 ml koala semen, stimulation of the urogenital sinus with a purposebuilt glass rod (designed to mimic the action of the penis during natural mating) and urogenital stimulation with the glass rod followed by insemination of 1 ml koala semen. Confirmation of a luteal phase was based on evidence of a prolonged return to oestrus, parturition and/or elevated progesterone concentrations. Insemination of saline (0/9) and urogenital stimulation (0/9) failed to induce a luteal phase. Insemination of semen without glass rod stimulation resulted in a luteal phase in 4/9 koalas, three of which gave birth. Insemination of semen in combination with urogenital stimulation produced a luteal phase in 7/9 koalas, four of which gave birth. Semen had a significant effect on induction of the koala luteal phase ($P < 0.001$) but glass rod stimulation had no such effect ($P = 0.335$). It was concluded that semen must be involved in the induction of a luteal phase in the koala. The results presented in this study will serve to improve optimal timing and induction of ovulation for artificial insemination in the koala.
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Introduction

The koala is the only known marsupial for which copulation is required to induce a luteal phase (Johnston *et al.* 2000a). In the absence of mating, oestrus persists for approximately 10 days, the corpus luteum does not form, plasma progesterone remains at basal concentrations and females return to oestrus about 33 days later. However, if the oestrous female receives a full copulatory stimulus from the male (Johnston *et al.* 2000b), the preovulatory follicle ovulates, a corpus luteum forms and there is a consequent rise in progestogen concentration; once mated, the female koala rarely continues to display oestrous behaviour. When fertilization is successful, parturition will occur after a gestation period of approximately 35 days. If fertilization is unsuccessful, the female will return to oestrus approximately 50 days after coitus. The longer oestrous cycle length accounts for both follicular and luteal phase durations, whereas the shorter cycle is represented by a period that only incorporates atresia of the dominant follicle and a subsequent follicular phase. Mating stimulus clearly induces the koala luteal phase (Johnston *et al.* 2000a, 2000b) and, by inference, ovulation. What is less clear is the mechanism by which the luteal phase is induced.

Studies by Johnston *et al.* (2000b) have shown that if koala females receive only a partial mating stimulus (approximately half of the thrusting period) then they will typically not enter a luteal phase. By contrast, females receiving a full copulatory stimulus show evidence of a luteal phase. Johnston *et al.* (2000b) have suggested that this may be evidence of a copuloceptive reflex in the koala, similar to that in other reflexovulating species such as the cat or rabbit; hence, female koalas receiving only half the thrusting stimulus have not received an adequate neural excitation of the urogenital system. However, there is also another explanation which has yet to be investigated and this involves the possibility that the mechanism for luteal phase induction is associated with the biochemical composition of inseminate, in a similar way to that reported in the bactrian camel (*Camelus bactrianus*;

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Chen *et al.* 1985; Xu *et al.* 1985). More recently, Pan *et al.* (2001) described the isolation and purification of an ovulation-inducing factor in the seminal plasma of the bactrian camel, indicating that the substance was completely different to native gonadotropin releasing hormone (GnRH), luteinizing hormone (LH), human chorionic gonadotropin (hCG), pregnant mares serum gonadotropin (PMSG) and prostaglandin F-2α. Interestingly, ovulation-inducing factor has also been shown to have GnRH-like activity in that it causes the release of LH and follicle-stimulating hormone (FSH) from cultured pituitary tissue and induces ovulation after injection into the mouse and camel (Pan *et al.* 2001). Similar factors have also been reported in the seminal plasma of alpacas (Sumar 2000), humans (Sokol *et al.* 1985) and the testis of the rat (Bhasin and Swerdloff 1984). The present study will attempt to determine the relative importance of coital and/or seminal stimulation to the ovulatory mechanism in the koala.

To date, evidence of the coital induction of the luteal phase in koala has primarily been based upon observations of a post-coital rise in progestogen and a prolonged oestrous cycle (Johnston *et al.* 2000a, 2000b); identification of a post-coital LH surge has not been described in the koala. Comparative studies that characterize LH surge in marsupials are limited to the Tammar wallaby (*Macropus eugenii*; Sutherland *et al.* 1980), kowari (*Dasyuroides byrnie*; Fletcher 1983) and brushtail possum (*Trichosurus vulpecula*; Fletcher and Selwood 2000). In *M. eugenii* (Sutherland *et al.* 1980), the LH surge occurs 8 h after oestrus and is followed by ovulation 24–48 h later. Unusually, *D. byrnie* (Fletcher 1983) females demonstrate a LH peak 12 days before oestrus; its significance to ovulation remains ambiguous. A pre-ovulatory surge of LH (maximum 25–30 ng/ml) is detected in *T. vulpecula* following oestrus and lasts less than 24 h (Fletcher and Selwood 2000).

While Johnston *et al.* (2003) have reported successful artificial insemination (AI) in the koala using fresh semen, further development of the insemination technique with extended or frozen semen will require a better understanding of the mechanism of ovulation and the timing of the LH surge in this species. The aims of this study, therefore, were to determine the timing of the LH surge with respect to coitus and the relative importance of coital stimulation and seminal plasma to the ovulatory mechanism in the koala.

Materials and Methods

Animals

All koalas (Lone Pine Koala Sanctuary, Brisbane, Australia) used in this study were sexually mature and clinically healthy during the course of the study. Experiments were conducted during the breeding season from January to March and September to November 2000. Koala husbandry, reproductive management and venipuncture have been described elsewhere (Blanshard 1994). Experimental protocols conducted in this study were approved by the University of Queensland's Animal Ethics Committee.

Experiment 1: timing of the LH surge in the koala

This experiment examined the copulatory stimulus necessary to induce an LH surge in the koala. During preliminary investigations three levels of stimulus (based on previous descriptions of mating behaviour; Johnston *et al.* 2000a) were applied to females on day 2 of oestrus: (1) natural copulation ($n = 3$), (2) mounting and neck-biting behaviour without penile penetration of the female's urogenital sinus ($n = 3$) and (3) the presence of a male without physical contact with female ($n = 3$). These koalas were bled to determine plasma LH concentration 1 h prior to receiving their treatment stimulus, immediately after the stimulus was applied and 1, 2, 4, 8, 12 and 24 h post-stimulus. Animals were monitored for parturition or return of behavioural oestrus (Blanshard 1994). Observations of subsequent oestrus and parturition confirmed a luteal phase in only those animals receiving the natural mating stimulus; there was no significant rise in LH concentration in any treatment group in the 24 h post-stimulus. Based on these preliminary observations, a further eight koalas were again naturally mated ($n = 4$) or exposed to the presence of the male without physical contact ($n = 4$) on day 2 of oestrus. Blood samples were recovered for determination of plasma LH concentration at −1, 0, 12, 16, 20, 24, 28, 32, 36 and 40 h post-coitus or introduction of the male. As in the preliminary experiment, koalas were monitored for parturition from day 34 to 37 and the length of their subsequent return to oestrus was determined.

Experiment 2: the mechanism of luteal phase induction in the koala

This experiment tested whether physical stimulation of the urogenital sinus and/or the introduction of semen in the female reproductive tract was responsible for induction of ovulation and therefore a luteal phase. Female koalas were allocated randomly into one control and three treatment groups ($n = 9$ per group). The control group (−S/−GR) received a saline infusion (1.0 ml) through a Cook Koala AI Catheter (Fig. 1A; Cook Pty Australasia, Brisbane, Australia; Johnston *et al.* 2003) that was inserted into the urogenital sinus as per the normal AI procedure. The first treatment group (+S/−GR; $n = 9$) was inseminated using the Cook catheter with approximately 1 ml koala semen that had been collected by artificial vagina (AV; Johnston *et al.* 1997). The koala ejaculate contains an aspermic or 'sperm-poor' fraction, which coagulates during mating to produce a copulatory plug, and a sperm-rich fraction. The sperm-rich fraction remains liquefied post-ejaculation and is used for AI; the volume of the spermrich fraction collected by AV typically measures 0.7 ± 0.1 ml (Johnston *et al.* 1997). The urogenital sinus of the second treatment group (−S/+GR; $n = 9$) was

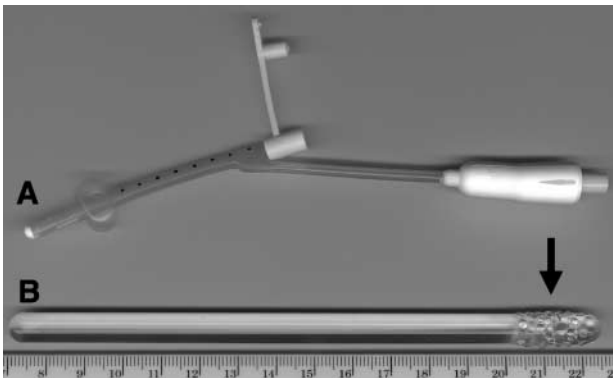

Figure 1 (A) Cook Koala AI catheter. (B) Glass rod used to stimulate the urogenital sinus of the female koala in experiment 2; arrow, note the stippled region on the terminal portion of the glass rod designed to mimic the cornified spines immediately distal to the koala glans penis.

manually stimulated with a purpose-built glass rod that mimicked the koala penis (Fig. 1B). The glass rod was worked back and forth along the length of the urogenital sinus with a slight twisting motion to a depth of approximately 40–60 mm; stimulation of the urogenital was based on previous descriptions of natural coitus (40 penile thrusts per 20 s; Johnston *et al.* 2000a, 2000b). The final treatment group (+S/+GR; $n = 9$) received urogenital stimulation with the glass rod, followed by AI of approximately 1 ml koala semen. All treatments were conducted during the breeding season on day 2 of the oestrous cycle. A luteal phase was confirmed by an elevated progesterone concentration on day 14 or day 28 greater than 0.7 ng/ml; this concentration represented the upper threshold progesterone concentration (99.99% confidence interval) from all 36 oestrus (day 2) koalas.

Hormone assays

Plasma LH was measured by heterologous RIA with antiserum GDN15 used as described by Curlewis (1991) with ovine LH as standard and radioligand. Koala pituitary extract and plasma were diluted in parallel with the standard. The sensitivity of the assay was 0.4 ng/ml, the maximum detection limit was 25 ng/ml and the intra-assay coefficient of variation was 12.6%. All samples reported in Fig. 2 were measured in the one assay. Progesterone concentrations in plasma extracts were measured by RIA as described by Curlewis *et al.* (1985) except that antiserum C-9817 (Bioquest, North Ryde, NSW, Australia) was used. Extraction efficiency was 74.8% and values reported here were not corrected for these losses. The sensitivity of the assay was 0.2 ng/ml and the intra- and inter-assay coefficients of variation were 5.6 and 8.0% respectively.

Statistical analysis

The results of experiment 2 where analysed as a 2×2 factorial design using an exact logistic regression technique (Collett 2002).

Results

Results from experiment 1 are shown in Fig. 2 and clearly indicate the presence of an LH surge in all four mated koalas approximately 24–32 h post-coitus (Fig. 2A). The mean (± S.E.M.) time of peak LH concentration following natural mating was 28.5 ± 1.7 h. The LH concentration of these animals remained above the lower detection limit of the assay for 7.0 ± 1.9 h. Peak concentrations of LH concentrations (>25.0 ng/ml) occurred for less than 4 h. All koalas that were mated naturally subsequently gave birth approximately 35 days later. For females that were introduced to males on day 2 of oestrus, but not permitted to mate, plasma LH concentrations remained below the detection limit of the assay for the entire sampling period (Fig. 2B). The oestrous cycle lengths of the four non-mated koalas were 28, 28, 30 and 40 days (31.5 ± 2.9 days). These cycle lengths are consistent with the lack of

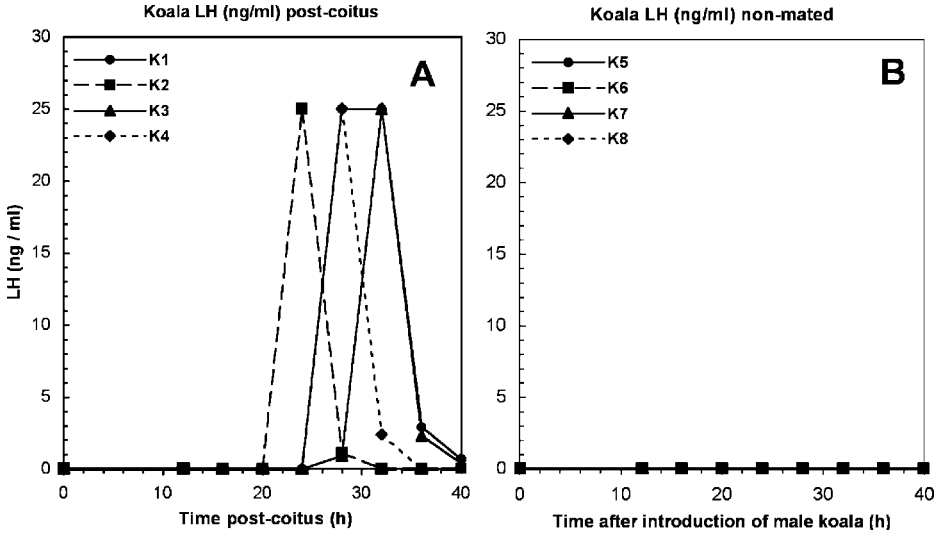

Figure 2 Plasma LH in female koalas that were (A) or were not (B) mated on day 2 of oestrus (experiment 1).

formation of a functional corpus luteum or luteal phase (Johnston *et al.* 2000a) and infer lack of ovulation in these animals.

Results of experiment 2 are presented in Tables 1 and 2. Table 1 documents the raw data for each treatment and for individual koalas. Table 2 is a summary of our interpretation of the data presented in Table 1. Insemination of sterile saline (−S/−GR) and glass rod stimulation (−S/+GR), failed to induce a luteal phase in any animal as progesterone concentrations remained less than 0.7 ng/ml at both day 14 and day 28. In contrast, insemination of semen without the glass rod stimulation (+S/−GR) resulted in a luteal phase in four out of nine koalas, as indicated by elevated progesterone concentrations, and three of these produced pouch young. Insemination of semen in combination with rod stimulation (+S/+GR) induced a luteal phase in seven of nine koalas, four of which subsequently gave birth. Based on the 2×2 factorial design and using an exact logistic regression technique, insemination with semen was shown to have a significant effect on induction of the koala luteal phase ($P < 0.001$) whereas stimulation with the glass rod had no such effect ($P = 0.335$). It was not possible to estimate the statistical interaction between the two treatments using exact logistic regression. However, a Fisher's exact test (Fleiss 1981) revealed no statistical difference ($P = 0.335$) in the rate of luteal phase induction between the +S/−GR (4/9) and the +S/+GR (7/9) treatment groups.

Discussion

The data presented in experiment 1 of this study are the first description of an LH surge in the koala and confirm the importance of coitus for induction of the LH surge. The LH surge occurred some 24–32 h post-coitus, which is much later than observed in other reflex ovulators. For example, in the camel (bactrian, Xu *et al.* 1985; dromedary, Marie and Anouassi 1986), rabbit (Dufy-Barbe *et al.* 1973; Goodman and Neill 1976), cat (Concannon *et al.* 1980; Wildt *et al.* 1980; Johnson and Gay 1981), vole (Charlton *et al.* 1975) and ferret (Carroll *et al.* 1985) plasma LH concentrations begin to increase within an hour of copulation, reach peak values within 1–6 h and return to baseline within 24 h; however, the duration

Table 1 Occurrence of oestrus, births and plasma progesterone (Prog) in individual koalas from experiment 2.

Treatment	Koala ID	Return of oestrus post treatment (days)	Progesterone (ng/ml)			Luteal phase induced
			Day 0 (oestrous)	Day 14	Day 28	
AI catheter only (−S/−GR)	K9	30	0	0	0	–
	K10	24	0	0	0	–
	K11	27	0	0	0	–
	K12	32	0	0	0	–
	K13	37	0	0	0	–
	K14	30	0	0	0	–
	K15	30	0	0	0	–
	K16	No data	0	0	0	–
	K17	33	0	0	0	–
Semen only (+S/−GR)	K18	No data	0	1.7	4	√
	K19	35	0	0	0	–
	K20	28	0	0	0	–
	K21	Birth	0.2	2.9	10.7	√
	K22	24	0	0	0.5	–
	K23	34	0	0	0	–
	K24	Birth	0	3.5	9.1	√
	K25	Birth	0	2.9	4.8	√
	K15	38	0	0	0	–
Glass rod only (−S/+GR)	K26	26	0.6	0.3	0.2	–
	K27	33	0.2	0.3	0.2	–
	K28	31	0	0	0	–
	K29	38	0.2	0.2	0	–
	K22	27	0	0	0	–
	K30	38	0	0	0.2	–
	K31	35	0	0	0	–
	K32	31	0	0	0	–
	K33	31	0	0	0	–
Glass rod + semen (+S/+GR)	K34	24	0.14	0.3	0.2	–
	K35	45	0	0.9	2.9	√
	K36	30	0	0	0	–
	K37	Birth	0	1.9	2.3	√
	K38	48	0	2.2	3.0	√
	K39	49	0.2	2.2	6.3	√
	K40	Birth	0	1.5	6.3	√
	K41	Birth	0	1.4	5.2	√
	K42	Birth	0	1.2	8.5	√

Table 2 Proportion of koalas in which treatments induced a luteal phase in experiment 2.

	Semen (S)	
Glass rod stimulation (GR)	−S	+S
−GR	0/9	4/9
+GR	0/9	7/9

of maximal LH secretion in these species is more variable, ranging from 2 h in the vole up to 12 h in the domestic cat. The much longer delay between copulation and the LH surge in the koala (24–32 h) is unusual for a coital or semen-induced ovulating species. This may have implications for the application of assisted reproductive technologies, as ovulation is also likely to occur much later than might have been expected. For example, if ovulation follows the LH surge in the koala by 24–48 h, as it does in *M. eugenii* (Harder *et al.* 1985), then the oocyte may not be available for fertilization until some 48–56 h post-coitus. Results from this study also suggest that the duration of elevated LH secretion was probably less than 4 h and in this respect formed a relatively sharp-spiked pattern of secretion compared to the more broad profiles of most other induced ovulating species, save the vole (Carroll *et al.* 1985).

Experiment 2 provides evidence to suggest that the introduction of semen into the urogenital sinus has an important role in the mechanism of luteal-phase induction in the koala; a role that is apparently more significant than that of glass rod mechanical stimulation (penile thrusting) in the urogenital sinus of the female. This result appears somewhat contradictory to that of an earlier study where we showed that the duration of thrusting during copulation had a significant effect on the induction of the luteal phase in the koala (Johnston *et al.* 2000b). In that study, we showed that successful induction of the luteal phase during natural coitus typically required the full thrusting period of the male to be completed. These results also showed that successful fertilization and pregnancy required at least half the ejaculation of the sperm-rich fraction. There are at least two possible explanations for this apparent inconsistency. The first of these might be explained by the fact that the glass rod stimulation protocol used in the current study was simply not suitable to induce an artificial copuloceptive reflex; alternatively the second explanation may be found in the timing and type of koala semen fractions that are ejaculated during coitus. Koala semen collected with an artificial vagina is usually recovered in two fractions (Johnston *et al.* 1997); a coagulated typically non-spermic fraction that is released during thrusting behaviour and which forms copulatory plug material on the AV liner, followed by a sperm-rich liquid fraction that is drained into the collection vial of the AV post-penile thrusting. If natural copulation and ejaculation in the koala are similar to that described in the AV, then it is possible that the first non-spermic fraction also has an ovulating factor, capable of inducting a luteal phase. In practice, it is also possible that sperm-rich semen might occasionally be ejaculated prematurely before termination of penile-thrusting behaviour.

The results of this study suggest that koala semen contains a factor or factors that promote induction of the luteal phase, and in this respect the mechanism of ovulation may superficially resemble that of the bactrian camel (Chen *et al.* 1985; Xu *et al.* 1985; Sumar, 2000; Pan *et al.* 2001). Whether semen alone is sufficient to induce ovulation in the dromedary camel is still debatable (Anouassi *et al.* 1992). Dromedary camels mated with a vasectomized male followed by AI using freshly diluted semen ovulated on 60% of occasions. Females inseminated with fresh or freshly diluted semen without coital stimulation by the male only ovulated 33 and 20% of the time, respectively. Similarly, in the current study, koalas receiving both glass rod stimulation and insemination of fresh semen had a higher (but not statistically significant) induction rate (7/9) than those inseminated with koala semen but without coital stimulation (4/9). Perhaps, the presence of specific ovulating factors in the semen and the mechanical stimulation of the urogenital sinus during coitus work synergistically to induce ovulation (El Wishy 1987). It is unlikely that stretching of the urogenital sinus associated with the increased seminal volume causes stimulation of stretch receptors and a subsequent neural reflex in the koala, as females inseminated with 1 ml sterile saline showed no evidence of a luteal phase.

The identification of a koala LH surge some 24–32 h post-coitus and the new knowledge that semen plays an important role in induction of the luteal phase are both strategic pieces of information in the development and refinement of an AI program in the koala. For example, frozen–thawed koala spermatozoa are likely to have a shortened survival time post-thaw and insemination closer to the time of ovulation may increase the likelihood of successful fertilization. Similarly, it may be important to use semen from a vasectomized male prior to AI in order to induce ovulation; this could be followed by insemination of frozen–thawed donor semen 48–56 h later. It is also possible that there may be a threshold dilution dose of koala semen in preparation for AI beyond which the diluted semen (and ovulation-inducing factor) is not capable of causing ovulation. While conducting experiment 2, a further seven koalas were produced by AI; this brings the total number of koalas born using AI by the current authors to 13. The AI techniques used in this study were all conducted on conscious animals using non-invasive procedures. The koala still remains the only species of marsupial born by assisted-breeding technology.

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