

# Wombat reproduction (Marsupialia; Vombatidae): an update and future directions for the development of artificial breeding technology

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## Abstract

This review provides an update on what is currently known about wombat reproductive biology and reports on attempts made to manipulate and/or enhance wombat reproduction as part of the development of artificial reproductive technology (ART) in this taxon. Over the last decade, the logistical difficulties associated with monitoring a nocturnal and semi-fossorial species have largely been overcome, enabling new features of wombat physiology and behaviour to be elucidated. Despite this progress, captive propagation rates are still poor and there are areas of wombat reproductive biology that still require attention, e.g. further characterisation of the oestrous cycle and oestrus. Numerous advances in the use of ART have also been recently developed in the Vombatidae but despite this research, practical methods of manipulating wombat reproduction for the purposes of obtaining research material or for artificial breeding are not yet available. Improvement of the propagation, genetic diversity and management of wombat populations requires a thorough understanding of Vombatidae reproduction. While semen collection and cryopreservation in wombats is fairly straightforward there is currently an inability to detect, induce or synchronise oestrus/ovulation and this is an impeding progress in the development of artificial insemination in this taxon.

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## Introduction

Within the Vombatidae, there are three species of wombat: *Vombatus ursinus* (common wombat, CW), *Lasiorhinus latifrons* (southern hairy-nosed wombat, SHNW) and *Lasiorhinus krefftii* (northern hairy-nosed wombat, NHNW). CW and SHNW have a current conservation status of 'least concern', but NHNW is 'critically endangered' (IUCN 2011). The NHNW is one of world's rarest species, with an estimated 138–160 individuals remaining in a remnant population in central Queensland (QLD) and 12 individuals recently reintroduced into an insurance population in southwest QLD (A Horsup 2012, personal communication, NHNW recovery team leader). Since European colonisation, there has been a substantial reduction in the distribution of all wombat species (Horsup 2004, Triggs 2009). The SHNW is now confined to four main population centres in South Australia (SA), while the CW has almost disappeared from the western half of Victoria (VIC), the Bass Strait Islands as well as from many parts of New South Wales (NSW) where it formerly ranged (Triggs 2009).

CW and SHNW have only been routinely kept in captivity since the 1970s, and NHNW are not currently held within any zoological institution (Jackson 2003). Historically, captive wombats have only bred occasionally and no sustainable breeding programme has ever been established; second generation (F2) breeding has yet to occur (V Nicolson 2012, personal communication, SHNW studbook keeper). When considered as one total population, the Australian *ex situ* wombat population is unsustainable. The mean annual recruitment rate (since 2003) has been 2.5, while the mean annual loss (via death, exports and releases) has been 7.3, and the population has only survived due to restocking with wild-born individuals (V Nicolson 2012, personal communication). Failure to breed wombats in captivity has been primarily associated with a lack of knowledge of their fundamental reproductive physiology and behaviour, combined with inappropriate husbandry and housing. Captive breeding programmes are likely to play an important role in wombat conservation and given their 'least concern' status and close phylogenetic relationship, the CW and SHNW are valuable

model species for the development of assisted breeding technologies in the NHNW.

Wombat nocturnalism, fossorialism, difficulties of field capture (for repeated sampling) and reticence to breed in captivity have hindered the study of reproductive biology in this taxon. Nevertheless, since 2002, significant advances have been made in characterising the oestrous cycle (West *et al.* 2004, Finlayson *et al.* 2006, Hogan *et al.* 2010b) and in understanding male seasonality and fertility (Hamilton *et al.* 2000, Taggart *et al.* 2005, Hogan *et al.* 2010a). While studies into the preservation of spermatozoa (Taggart *et al.* 1998, 2005, MacCallum & Johnston 2005) and manipulation/control of the oestrous cycle (West 2002, Drury *et al.* 2007, Finlayson *et al.* 2007b) have been attempted, there are still many aspects of wombat reproduction that require further characterisation. The aim of this review is to provide an update on the current knowledge of wombat reproductive biology (highlighting species differences) and to identify where additional research is required in terms of developing reliable artificial breeding technology. Given that there is very limited information on NHNW reproduction, this review will primarily focus on the CW and SHNW.

## Reproductive anatomy and morphology

Table 1 reports a summary of the studies on male and female reproduction in the Vombatidae.

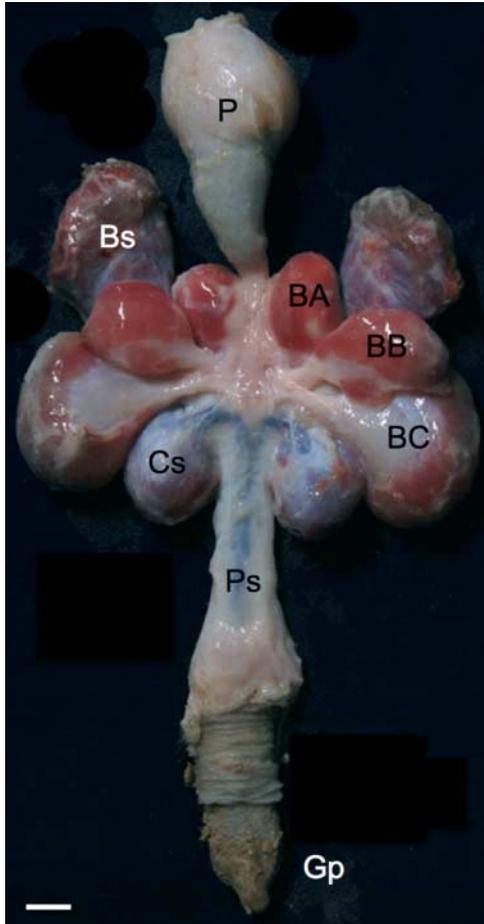
## Male gross anatomy

The gross anatomy of a male CW reproductive system is shown in Figs 1 and 2. Wombat testes are held in a non-pendulous pre-penile scrotum (Fig. 2A), are ellipsoidal in shape and enclosed (with the epididymides) within a non-pigmented tunica vaginalis (Fig. 2B). The epididymis has three distinctive segments (caput, corpus and cauda) with the caput epididymis being loosely attached to the dorsal pole of the testes and cauda epididymis enclosed within a pocket of the tunica vaginalis (Fig. 2C and D). The vas deferens is non-specialised, possesses no ampullae and empties into the prostatic urethra. The disseminate prostate is enclosed in a fibromuscular capsule, is carrot-shaped and consists of three distinct segments (anterior, central and posterior) with the divisions running transversely (Fig. 2E). While there are no seminal vesicles, there are three pairs (A, B and C) of bulbourethral glands (Fig. 1) in close association with the penile crura and urethral bulbs. The flaccid penis is held in an S-bend configuration within a preputial sac adjacent to the cloacal opening and when erect, it is ~100 mm in length. While the glans penis is bifurcated, the urethra terminates at the base of this bifurcation but extends as a urethral groove along the inner medial aspects of each bifurcation. The penile shaft has small cuticular spines, ~20 mm distal to the glans penis. The functional significance of these spines is unknown. While penile spines in other mammals (e.g. felids) are sometimes associated with vaginal or cervical stimulation resulting in induced ovulation, there is currently

**Table 1** Published information on Vombatidae reproductive anatomy.

References	Species	Sex	Anatomical content
Shaw (1800)	CW	♂	General gross anatomy, including reproductive organs
Home (1836)	CW	♀	Reproductive anatomy (anecdotally reported)
Owen (1845)	SHNW	♂	General gross anatomy (brief comments on reproductive organs)
O'Donoghue (1916)	CW	♀	Description of the appearance of the ovaries and ovarian interstitial tissue
MacKenzie & Owen (1919)	CW	♂	Detailed account of the gross anatomy of the reproductive system
		♀	Full description of the gross urogenital anatomy
Pearson (1944)	CW	♀	Description of the gross urogenital anatomy
Barnett & Brazenor (1958)	CW	♂	Presence of a rete mirabile in the spermatic cord
Hughes (1965)	CW	♂	Sperm morphology
Brooks <i>et al.</i> (1978)	SHNW	♂	Full description of the reproductive system; structural and biochemical characteristics of accessory reproductive glands; sperm morphology
Barbour (1981)	SHNW	♂	Full description of the reproductive system; histology and histochemistry of accessory reproductive glands
Gaughwin (1981)	SHNW	♂	Structural and biochemical characteristics of the reproductive organs
		♀	Structural characteristics of the urogenital tract
Hughes & Green (1998)	CW	♀	Placentation
Moritz <i>et al.</i> (1998)	CW	♀	Ovarian and uterine histological changes associated with the oestrous cycle
Taggart <i>et al.</i> (1998)	SHNW	♂	Seminal characteristics
Hamilton <i>et al.</i> (2000)	SHNW	♂	Seasonal change in accessory glands (structure)
Breed <i>et al.</i> (2001)	CW	♂	Sperm morphology (i.e. the structural organisation of sperm head components)
MacCallum (2004)	CW	♂	Gross anatomy and testicular histology of the reproductive tract and sperm morphology
		♀	Examined the urogenital system (i.e. for the development of an AI protocol)
Taggart <i>et al.</i> (2005)	SHNW	♂	Seasonal changes in the testis, accessory glands and ejaculate characteristics
Hogan <i>et al.</i> (2010a)	SHNW	♂	Seasonal changes in prostate and bulbourethral gland size

CW, common wombat (*Vombatus ursinus*); SHNW, southern hairy-nosed wombat (*Lasiorhinus latifrons*); NHNW, northern hairy-nosed wombat (*L. krefftii*); AI, artificial insemination.



**Figure 1** Dissected reproductive tract of a male wombat (*Vombatus ursinus*). White scale bar = 10 mm. BA, bulbourethral gland A; BB, bulbourethral gland B; BC, bulbourethral gland C; Bs, bulbospongiosum; Cs, crus penis; Gp, gland penis; P, prostate; Ps, penis.

no evidence to support this pattern in the wombat (Paris *et al.* 2002, West *et al.* 2004, Hogan *et al.* 2010b).

### Male micro-anatomy

#### Testis

Surprisingly, there has been little description of the micro-anatomy of the wombat testis. Barnett & Brazenor (1958) noted the presence and fine structure of a rete mirabile in the CW spermatic cord, which was later reconfirmed by MacCallum (2004). The seminiferous epithelial cycle and micro-anatomy of the CW (MacCallum 2004) and SHNW (Oishi *et al.* 2013) testis have been described. Both species have similar seminiferous tubule mean diameters (CW:  $234.0 \pm 4.6 \mu\text{m}$ , SHNW:  $243.5 \pm 3.9 \mu\text{m}$ ), possess a high proportion of pre-meiotic (CW: 61.7%, SHNW: 66.6%) compared with post-meiotic stages of the seminiferous cycle and have a frequency of cellular associations and dynamics similar to those in other marsupials (Setchell & Carrick

1973, Lin *et al.* 2004, Phillips *et al.* 2008). Interestingly, round spermatid nuclei in wombatids (i.e. koalas and wombats) pass through an elongation phase in late stage I of the cycle to produce a 'rod shaped' uncondensed spermatid nuclei; this morphological stage has not been observed in other marsupials (Oishi *et al.* 2013). Although a close phylogenetic relationship exists between the koala and the wombat, there are differences in testicular histology between the two species, with the koala displaying a greater proportion of Leydig cells, larger Sertoli cell nuclei, the presence of crystalloids in the Sertoli cell cytoplasm and a highly eosinophilic acrosome (Oishi *et al.* 2013).

**Sperm morphology and spermiogenesis.** Hughes (1965) revealed that wombatid spermatozoa were murid-like in morphology and unique amongst the marsupials; the sperm head is 'sickle-shaped' and the neck of the flagellum inserts disto-ventrally. Although the overall sperm head shape of the koala and wombat are similar in form, there are small inter-species differences in the chromatin structural organisation (Breed *et al.* 2001). Nevertheless, both species have an acrosome that lies within the concavity of the nucleus and from this region a thin segment of acrosome runs both posteriorly and laterally (Breed *et al.* 2001). Another unusual feature of wombatid spermatozoa is the large degree of heterogeneity in sperm head shape. In the CW this heterogeneity has been defined into six morphotypes and varies from a tight almost circular hook, to a straight or slightly dorsally recurved rod shape (MacCallum 2004). Wombatids also appear to show some heterogeneity in terms of their acrosome morphology (Temple-Smith 1994). Although spermiogenesis in wombatids follows a basic marsupial pattern (Temple-Smith 1994), there are a number of distinctive processes that include: formation of a pro-acrosomal granule within the acrosomal vacuole; an uneven condensation of chromatin; a flattening of the nucleus and a ventral neck insertion of the mid-piece into the sperm head, which is thought to account for the variation in sperm head morphology (Brooks *et al.* 1978, Harding *et al.* 1987, Harding & Aplin 1990, Oishi *et al.* 2013).

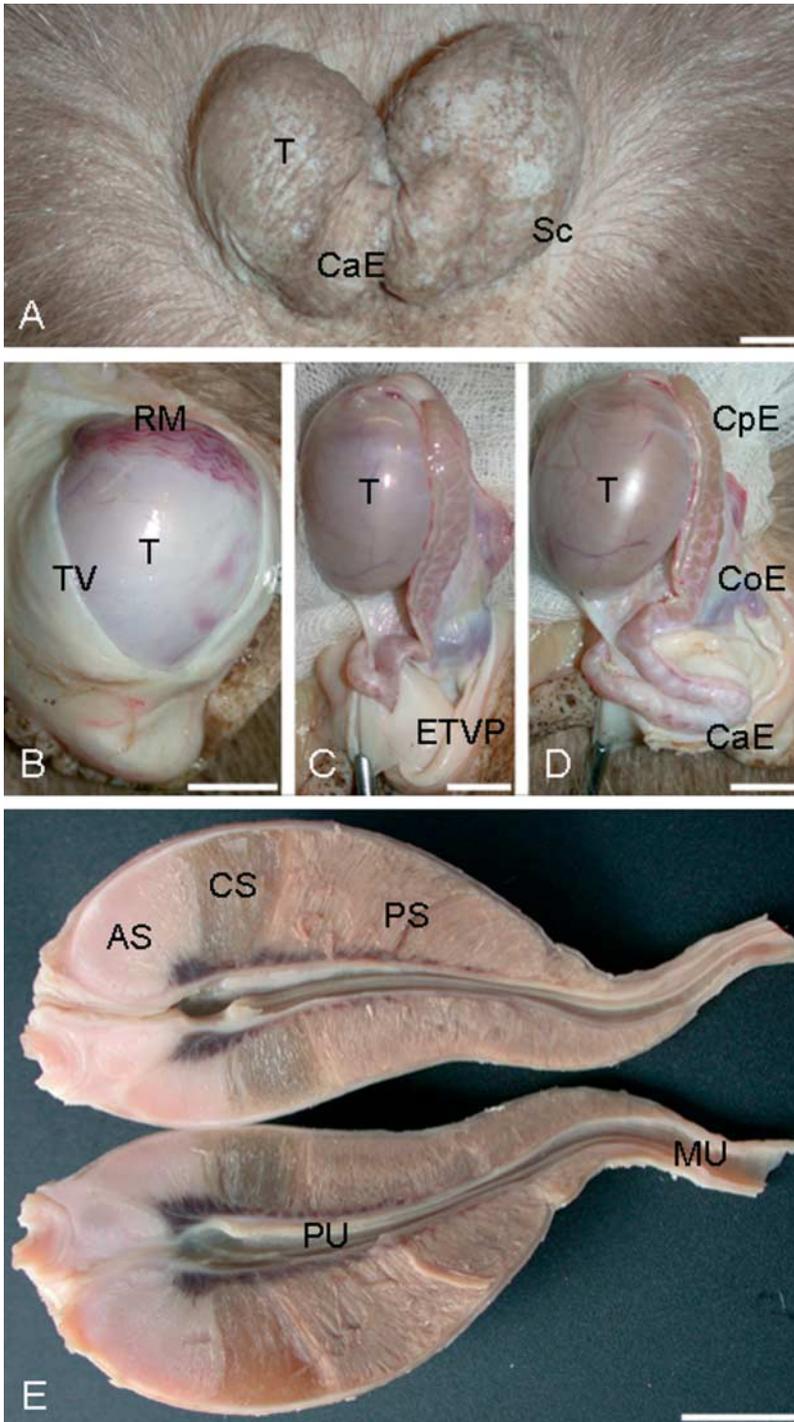
**Epididymis and sperm maturation.** There is currently little information on the gross anatomy and no information on the micro-anatomy of the wombat epididymis. The gross anatomy of the CW epididymis has been described by MacKenzie & Owen (1919) and MacCallum (2004), but there is only one observation for the SHNW (Brooks *et al.* 1978). All three studies reported that the cauda epididymis was enclosed within a separate pocket of the tunica vaginalis but no functional significance for this structure has been provided. Wombat spermatozoa are immature when they enter the head of the epididymis and are characterised by the head being only slightly recurved or of an irregular spiral configuration (Brooks *et al.* 1978); during epididymal maturation there is a marked increase in the recurvature of the sperm head,

condensation of the accessory cytoplasmic droplet within the hook of the sperm head and rotation of the long axis of the head parallel to that of the flagellum (Hughes 1965). During epididymal transit, wombat spermatozoa show no structural changes to their plasma membrane or cytoskeleton in the mid-piece region; this is in contrast to other marsupial spermatozoa, including

to a limited degree the koala (Harding *et al.* 1979, Harding & Aplin 1990, Temple-Smith 1994).

*Accessory glands*

The segments of the wombat prostate differ in their cytology, tubular histology and histochemistry, and



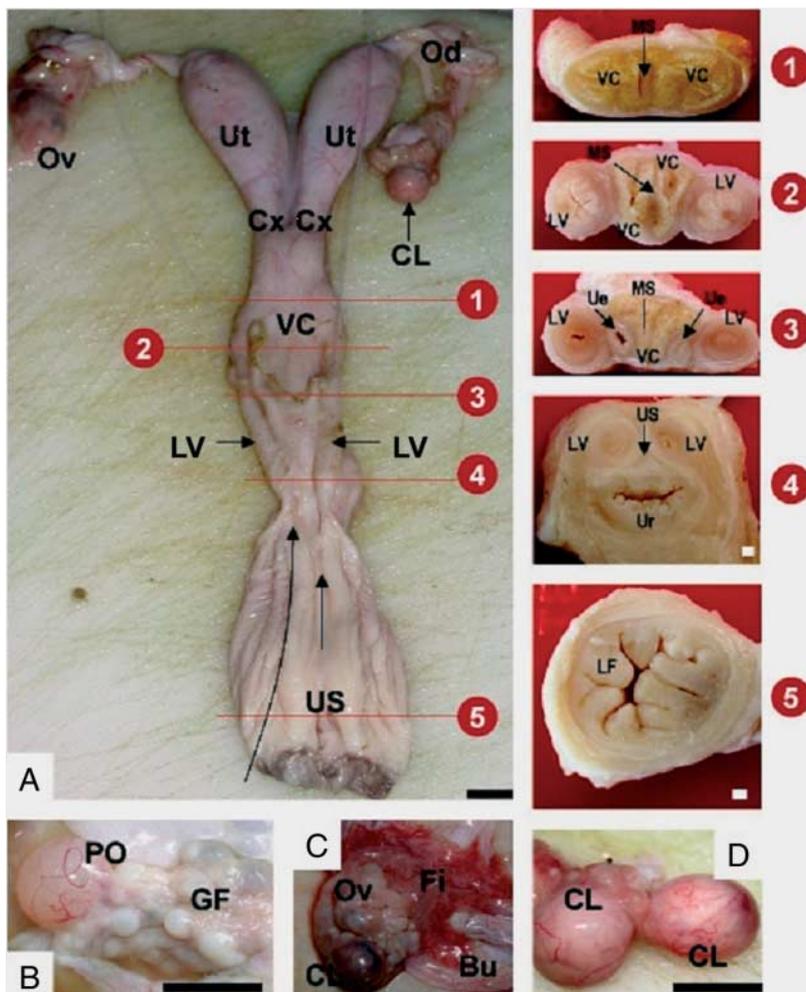
**Figure 2** Gross reproductive anatomy of a male wombat (*Vombatus ursinus*). (A) Non-pendulous scrotum; (B) testis; (C) testis and tunica vaginalis; (D) testis and epididymis; and (E) internal organisation of the prostate. White scale bars = 10 mm. AS, anterior segment; CS, central segment; CaE, cauda epididymis; CoE, corpus epididymis; CpE, caput epididymis; ETVP, epididymal tunica vaginalis pouch; MU, membranous urethra; PS, posterior segment; PU, prostatic urethra; RM, rete mirabile; Sc, scrotum; T, testis; TV, tunica vaginalis. Adapted, with permission, from MacCallum (2004).

secretory products (see review by Barbour (1981)). Prostatic bodies (i.e. secretory globules) occur in the glandular lumen of all segments but are particularly evident in the posterior segment (Brooks *et al.* 1978). Sorbitol, fructose and glycogen are produced by all three prostate segments, whereas only the central and posterior segments produce glucose and *N*-acetylhexosamine (Brooks *et al.* 1978, Tyndale-Biscoe & Renfree 1987). Iron is another major secretory product of the posterior segment, and acid mucin is also produced by the central segment (Barbour 1981). Wombat bulbourethral glands (three pairs) contain long branched, wide tubular glands surrounded by a layer of connective tissue and skeletal muscle (Brooks *et al.* 1978). Glands A and B are histologically identical except for the absence of interstitial mast cells from gland B; gland C is characterised by narrower tubules and larger epithelial cells (Barbour 1981). Gland A secretes primarily protein, gland B a neutral glycoprotein and gland C a sialomucin; glands B and C also contain cytoplasmic glycogen in their secretory cells (Barbour 1981).

### Female gross anatomy

The wombat has a rear opening pouch and the teats are reduced to a single pair (Triggs 2009). The juvenile pouch is shallow, with a depth of  $\sim 10$  mm and the teats inverted. The adult pouch is  $\leq 50$  mm in depth (often more deep laterally on one side), with teats in non-lactating females protruding to a length of 5 mm (Green & Rainbird 1987). There has been no description of the wombat mammary gland, nor has the chemical composition of wombat milk been analysed. Mammary gland development only occurs on the teat that is suckled and the teat itself can distend up to 85 mm long during lactation (Green & Rainbird 1987). To accommodate the increasing size of the developing pouch young (PY), the pouch enlarges anteriorly, laterally and posteriorly; after weaning, the pouch and recently suckled teat regress (Green & Rainbird 1987).

Figure 3 shows a dissected female CW reproductive tract from MacCallum (2004). The ovaries are ellipsoidal, dorso-ventrally flattened and enclosed (with the



**Figure 3** (A) Dissected urogenital system of post-luteal female wombat (*Vombatus ursinus*) with cross sections identifying the relative positions and internal luminal morphology of the different regions of the reproductive tract; (B) pre-ovulatory follicle; (C) corpus luteum; (D) two similar sized corpus luteums on the same ovary. White scale bars = 1 mm. Black scale bars = 10 mm. Bu, bursa; CL, corpora lutea; Cx, cervix; Fi, fimbriae; GF, Graafian follicle; LF, lateral folds of the urogenital sinus; Lv, lateral vagina; MS, medial septum; Od, oviduct; Ov, ovary; PO, pre-ovulatory follicle; Ue, ureter; Ur, urethra; Us, ovulatory strand; US, urogenital sinus; Ut, uteri; VC, vaginal culs-de-sac. Adapted, with permission, from MacCallum (2004).

infundibulum and part of the oviduct) within an ovarian bursa. There are two uteri, each with a distinct upper glandular endometrium and lower muscular cervix (Fig. 3A). Both cervixes extend caudally into the vaginal cul-de-sac, which is divided by a medial septum (Fig. 3A). The lateral vaginae enter the upper region of the vaginal cul-de-sac ~20 mm below the cervical ostia. The caudal portions of the lateral vaginae narrow significantly as they enter the cranial portion of the urogenital sinus. The bladder is connected by a short urethra that opens into the urogenital sinus behind the caudal extremity of the vaginal cul-de-sac. A dorso-ventrally flattened, bifid clitoris protrudes from the ventral floor of the urogenital sinus into the cloaca and both the urogenital sinus and rectum open up into the cloaca.

### Female ovarian micro-anatomy and folliculogenesis

The wombat ovary is ~20 mm in length by 10 mm in width, depending on the structures present during the different stages of the ovarian cycle (Fig. 3A and C; MacKenzie & Owen 1919, MacCallum 2004); it is ovoid, dorsally flattened and lobulated in appearance (Fig. 3A and C). The follicular dynamics of the CW ovary have been partially described by O'Donoghue (1916) and Moritz *et al.* (1998). O'Donoghue (1916) described the micro-anatomy of the mature Graafian follicle and observed that the theca interna of the ovarian follicle was made up of elongated polygonal cells 3–4 layers thick, similar to that of eutherian follicles. Moritz *et al.* (1998) attempted to link follicular development to ovulation and related changes in ovarian structures to the histology of the rest of the reproductive tract; despite using over 34 reproductive tracts, they were unable to document a female in oestrus or a pre-ovulatory follicle. MacCallum (2004) examined the ovaries of both anoestrous and post-luteal reproductive tracts and noted the occurrence (but not the size) of pre-ovulatory follicles and corpora lutea (Fig. 3B, C and D).

## Pattern of reproduction

### Reproductive strategy

Wombats have a conservative breeding strategy; there are two teats in the pouch but usually only one young is produced. Gestation is short (CW: 30–33 days (Green & Rainbird 1987), SHNW: 20–22 days (Crowcroft & Soderlund 1977)) and occupies a period less than the length of the oestrous cycle (Table 2). Pouch life is long and permanent emergence occurs at 10 months in the CW and at 6–9 months in the hairy-nosed wombats (HNWs) with weaning at 11–13 months (Gaughwin 1981, Boer 1998, Horsup 2004). Female wombats can produce one young in alternate years, the level of reproduction is strongly dependent on annual rainfall and forage availability. CWs and SHNWs become sexually mature at 2–3 years (McIlroy 1973, Gaughwin 1981), while maturity occurs at  $\geq 3$  years in the NHNW (Johnson 1991). The wombat reproductive strategy includes polyoestry, spontaneous ovulation and male/female polygyny (Jackson 2003).

### Timing of breeding

The probability of female SHNWs carrying PY is strongly correlated with plant growth in the previous 6-month period (Gaughwin *et al.* 1998) and the onset of reproduction is similarly associated with new plant growth (Gaughwin 1981). Likewise, the breeding rate of NHNWs is related to summer rainfall, with most females failing to breed in years with low rainfall (Crossman *et al.* 1994). These observations suggest a nutritional constraint on female breeding in the HNWs. The precise factors that control male HNW seasonality have not yet been identified. Several studies have demonstrated that peak male reproductive status coincides with the onset of female cyclicality, suggesting that male reproductive activity is activated in response to the female, or that the same environmental cue(s) control both sexes (Gaughwin *et al.* 1998, Taggart *et al.* 2005). A widely accepted hypothesis is that the wombat times its breeding so that the weaning of PY coincides with the maximum

**Table 2** Wombat oestrous cycle characteristics: a summary of published information.

Species	Mean cycle length (days)	Mean LP length (days)	Mean FP length (days)	Measured by	References
CW	33.0 (32–34)	–	–	Urogenital cytology	Peters & Rose (1979)
	55.1 (39–61)	28.2 (18–37)	25.6 (12–30)	Faecal progesterone metabolites (RIA)	Paris <i>et al.</i> (2002)
	47.2 (35–60)	27.8 (18–38)	18.8 (5–27)	Plasma progesterone	West <i>et al.</i> (2004)
	45.2 (32–55)	–	–	Vaginal cytology	West <i>et al.</i> (2004)
	50.7 (44–57)	–	–	Plasma progesterone	MacCallum (2004)
SHNW	50.4 (46–56)	–	–	Faecal progesterone metabolites (EIA)	MacCallum (2004)
	41.1	27.6	13.3	Faecal progesterone metabolites (RIA)	Paris <i>et al.</i> (2002)
	35.6 (35–38)	23.3 (16–26)	11.3 (10–12)	Plasma progesterone	Finlayson <i>et al.</i> (2006)
	31.8 (27–38)	20.9 (15–31)	11.6 (9–15)	Faecal progesterone metabolites (EIA)	Hogan <i>et al.</i> (2010b)

LP, luteal phase; FP, follicular phase; EIA, enzyme-immunoassay; CW, common wombat (*Vombatus ursinus*); SHNW, southern hairy-nosed wombat.

potential growth period of temperate grasses (Mallett & Cooke 1986, Jackson 2003, Taggart *et al.* 2005).

NHNWs have a clearly defined breeding season with 83% of births occurring during the summer months (December–February; Crossman 1988). SHNWs breed a little earlier, with a distinct breeding period between July and December, with most births occurring in October (Gaughwin *et al.* 1998). Breeding in the CW appears to be much less seasonal with some populations producing young year round. Within their distribution CWs appear to change the time in which females breed, with mating occurring later in the more southerly regions. As such, the majority of young are born during March–June in northern VIC (Nicolson 1963); June–July in Southern VIC (Presidente 1982); December–March in the highlands of NSW (McIlroy 1973); year round in Tasmania (TAS; Green & Rainbird 1987) and year round in eastern VIC, although a peak does occur in summer (West 2002, MacCallum 2004). There are currently no available reproductive seasonality data from the most northern population of CW located in Girraween National Park on the QLD/NSW border.

### **Breeding dispersal**

A common phenomenon amongst the Vombatidae is female-biased dispersal (Johnson & Crossman 1991, Banks *et al.* 2002, Walker *et al.* 2008). It is proposed that dispersal of adult females after breeding is an indirect form of maternal care, in that bequeathal of the natal burrow to the offspring increases the likelihood of its survival. While there appears to be ecological factors or phylogenetic constraints promoting female dispersal in wombats, male philopatry is common. Male wombats are more genetically similar to local rather than distant males and likely to have been born in the locale in which they are found (Taylor *et al.* 1997, Banks *et al.* 2002, Walker *et al.* 2008); this suggests that the incidence of dispersal by adult or juvenile males is negligible.

### **Male reproductive cycle**

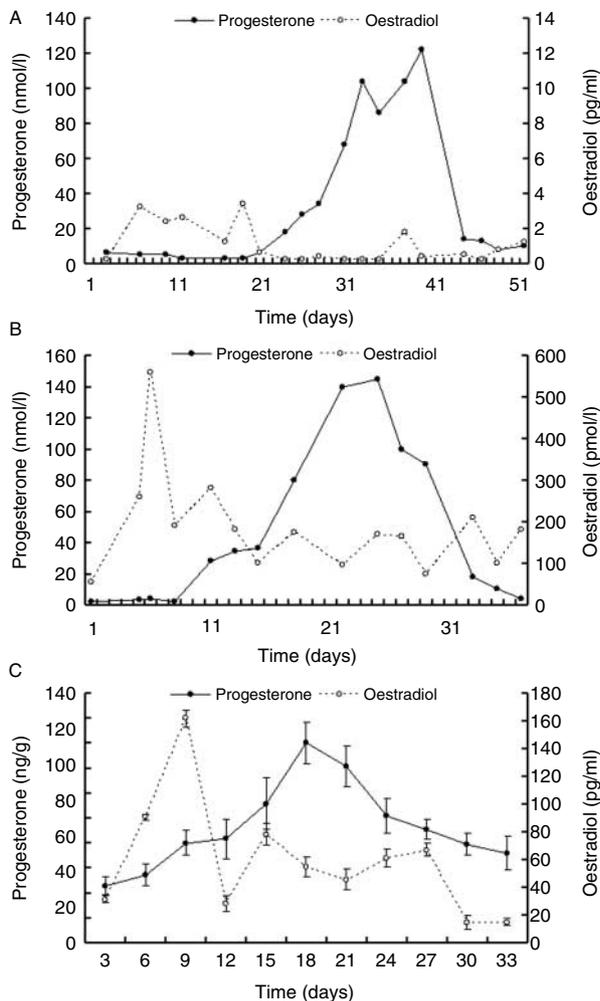
The CW and SHNW display substantive differences in the degree of male reproductive seasonality. A field study by McIlroy (1973) revealed seasonal changes in CW testicular diameters, with testes being larger during September–December. MacCallum (2004) found no seasonal variation in body weight, testosterone secretion, testicular volume or sperm quality in either wild or captive CWs. Although not statistically significant, MacCallum (2004) did note that wild CW accessory gland weights were higher in the breeding season when compared with those measured in the non-breeding season. A lack of a seasonal pattern of reproduction in male CWs is consistent with previous reports of births occurring year round (Green & Rainbird 1987).

In contrast, wild SHNW males undergo distinct seasonal changes in body weight, semen quality, testicular and accessory gland weight, and androgen concentration (Gaughwin 1981, Taggart *et al.* 1998, 2005, Hamilton *et al.* 2000); male reproductive function peaks in August–September and is followed by a marked decline in November. Druery (2004) was the first to monitor the reproductive status of captive SHNW males and discovered that androgen production increased between July and September, but there was no obvious change in testicular size. More recently, Hogan *et al.* (2010a) provided a more thorough account of captive male SHNW reproductive function and seasonality. Six different reproductive parameters were examined (for 18-months) and a significant seasonal change was detected in plasma testosterone secretion and bulbourethral gland size. Hogan *et al.* (2010a) concluded that the extent of captive male seasonality was less pronounced than that previously reported for wild SHNWs.

### **Female reproductive cycle**

Studies on the female reproductive cycle have shown that wombats are polyestrous, with females exhibiting at least to 2–3 cycles per breeding season (West *et al.* 2004, Finlayson *et al.* 2006, Hogan *et al.* 2010b). Oestrous cycle progesterone and oestradiol profiles have been described for the CW and SHNW (Fig. 4A, B and C). There is only one progesterone peak per cycle, during the luteal phase (LP; Fig. 4A and B), and this peak occurs 17–18 days after oestrus in the CW (West 2002) and 3–9 days after mating in the SHNW (Hogan *et al.* 2010b). Progesterone secretion/elevation without mating has been frequently observed, confirming spontaneous ovulation in this species (Paris *et al.* 2002, West *et al.* 2004, Hogan *et al.* 2010b). Oestradiol peaks (if observed) occur 3–8 days before the start of the LP and remain elevated for ~8 days (Fig. 4A; West 2002, Finlayson *et al.* 2006).

Wombat oestrous cycle length has been well documented (Table 2). In the CW there seems to be a high degree of natural variation between individuals in cycle length and Moritz *et al.* (1998) have suggested that this is due to a positive correlation with body weight, i.e. as body weight increases so does cycle length. In the SHNW the LP represents ~60% of the oestrous cycle. A range of studies have found that changes in urogenital cytology, pouch and genitalia morphology and body temperature cannot be used to accurately determine oestrous cycle length, nor indicate the phase of the cycle or the timing of oestrus in wombats. The use of cytology to define the oestrous cycle is problematic as the anatomy of the urogenital sinus makes it difficult to be consistent when swabbing cells from the epithelium of the sinus wall (MacCallum 2004, Finlayson *et al.* 2006) and sinus length varies between individuals and within



**Figure 4** Wombat oestrous cycle progesterone and oestradiol profiles: (A) denotes a cycle from one common wombat (*Vombatus ursinus*) using plasma hormone measurement. Data from West (2002); (B) denotes a cycle from one southern hairy-nosed wombat (SHNW; *Lasiorchinus latifrons*) using plasma hormone measurement. Data from Finlayson *et al.* (2006); (C) denotes the mean ( $\pm$  S.E.M. bars) cycle ( $n=12$ ) from six SHNWs (*L. latifrons*) using faecal hormone measurement. Data from Hogan *et al.* (2010b). Day 1 is the first day of basal progesterone concentrations following the previous luteal phase.

an individual at different stages of the cycle (West *et al.* 2004). In the case of pouch condition: depth, opening size, wall thickness, degree of cleanliness and/or moisture, and teat length can all provide clues to the reproductive status of the female (i.e. whether cycling or not) but there are no distinct variations directly associated with the oestrous cycle (Peters & Rose 1979, West 2002, MacCallum 2004). The clitoris and pericloacal region can become swollen and tumescent during different stages of the cycle (Gaughwin 1982, West 2002, Finlayson *et al.* 2006), but this technique for assessing changes in reproductive status is not reliable, with some researchers not being able to detect any noticeable genitalia changes (MacCallum 2004). The

limited scale and small number of replicates used by Peters & Rose (1979) prevented the drawing of any strong conclusions as to whether body temperature rhythms could be correlated with cyclicity in the CW.

Currently, there is no reliable method for detecting oestrus in wombats. Although several behavioural studies have reported that female wombats become very active at oestrus (McIlroy 1973, Peters & Rose 1979, Triggs 2009), a study by Hogan *et al.* (2010b) indicated that oestrus was not detectable in female SHNWs using continuous observations of physical activity (via movement-sensitive transmitters), as there was no difference in the amount of physical activity recorded during oestrus and anoestrous, nor was there any correlation found between physical activity and the occurrence of reproductive behaviour. Further studies into reliable methods of oestrus detection are urgently required as this specific lack of information has probably been the most significant impediment to successful captive breeding management.

While there has been a significant advance in knowledge of the wombat oestrous cycle over the last decade, there are numerous hormonal, morphological and behavioural aspects of the cycle that are still poorly understood; luteinizing hormone (LH) or follicle-stimulating hormone (FSH) profiles have yet to be documented as has their respective relationship to natural follicular maturation and ovulation. There have also been no definitive studies that link elevated oestradiol concentrations to oestrus behaviour in wombats and as such the factors controlling oestrus are essentially unknown.

### Reproductive behaviour

Even though numerous studies have examined *Vombatidae* reproductive behaviour (Table 3), oestrus has rarely been observed and appears to be exceptionally short. Peters & Rose (1979) reported a period of 15 h during which mating occurred in the CW, while Hogan *et al.* (2010c) recorded a period of receptivity of  $\leq 13$  h in the SHNW. The period of female receptivity typically lasts 24–48 h in most mammals (Kilen & Schwartz 1999) and the reason why it is so short in wombats has yet to be determined. Peters & Rose (1979) first described the oestrus behaviour of wombats as very active (pacing) and aggressive, and since then only two other observations have been published (CW: Boer 1998, SHNW: Hogan *et al.* 2010c). Both studies reported that the oestrus female attracts the male's attention through a series of pre-copulatory behaviours termed 'attraction actions'. These actions include: 'nudging', where the female makes repeated nose-to-nose, nose-to-flank or flank-to-flank contacts with the male; 'rump presentation' where the female presents her urogenital region to the male; and 'frontal displays' where the female digs frantically and/or urinates frequently in front of the

**Table 3** Published information on Vombatidae reproductive behaviour.

References	Species	Behavioural content
Wells (1971)	SHNW	Captive observations of reproduction ( $n=5$ )
McIlroy (1973)	CW	Breeding-season-related changes in female behaviour (field study)
Crowcroft & Soderlund (1977)	SHNW	Captive observations of reproduction ( $n=3$ )
Gaughwin (1978)	SHNW	Following and copulatory behaviour of captive wombats ( $n=13$ )
Wells (1978)	SHNW	Field observations of male pursuit (of females) and sexual-related vocalisations
Gaughwin (1979)	SHNW	Description of flehmen in a captive male ( $n=1$ )
Brown & Young (1979)	CW	Male flehmen behaviour and mating in a pair of captive wombats ( $n=2$ )
Peters & Rose (1979)	CW	Intermittent observations of reproductive behaviour in captivity; oestrus behaviour
Gaughwin (1982)	SHNW	Captive observations of reproduction in wombats at Brookfield Zoo
Taylor (1993)	CW	Chase sequences and mating positions of free-ranging wombats
Boer (1998)	CW	Full description of reproductive behaviour in a pair of wombats at Hannover Zoo ( $n=2$ )
Marks (1998)	CW	Courtship and mating in a pair of free-ranging wombats ( $n=2$ )
Paris <i>et al.</i> (2002)	CW	Flehmen and sniffing behaviour of male wombats at University of Melbourne
Stenke (2002)	NHNW	Evidence of courtship in a pair of wombats ( $n=2$ )
Hughes & Hughes (2006)	CW	A field observation of daytime courtship and mating in a pair of wombats ( $n=2$ )
Triggs (2009)	CW	General description of reproductive behaviour in this species
Hogan <i>et al.</i> (2010c)	SHNW	Full description of reproductive behaviour in a captive population ( $n=12$ )

CW, common wombat (*Vombatus ursinus*); SHNW, southern hairy-nosed wombat (*Lasiorhinus latifrons*); NHNW, northern hairy-nosed wombat (*L. krefftii*).

male. Whether these behaviours are indicative of oestrus has yet to be determined, but their repetitive nature and high frequency of occurrence just before mating suggests that this is likely.

Comprehensive descriptions of the full suite of wombat courtship-to-mating behaviour are available for the CW (Boer 1998, Marks 1998) and SHNW (Hogan *et al.* 2010c) and will not be reiterated here. Interestingly, the courtship-to-mating ritual in both species is almost identical and can be divided into six consecutive phases: investigation, attraction, chase, restraint, coitus and recovery. Other similarities include: a long duration of courtship before and guarding following mating; mating being preceded by high levels of male sniffing, parades and pursuits; most mating occurs at night and is carried out within an enclosed space (Table 3). Given the short duration of oestrus and predominate nocturnal mating activity, confirmation of mating success or detection of oestrus for the purposes of artificial insemination (AI) is going to be very challenging in this species; i.e. reliable oestrus detection is going to require 24-h video surveillance along with a teaser male.

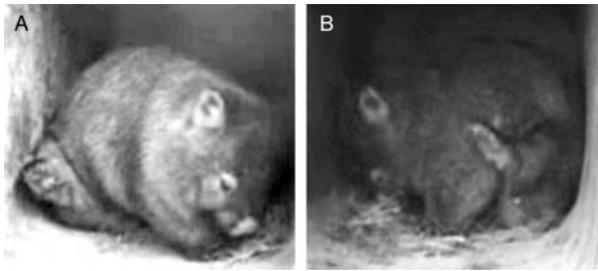
Several Australian zoos have an active wombat breeding programme but as yet, none have been able to establish a reliable, self-sustaining programme. Although pairs mate regularly, reports of resultant PY are infrequent suggesting low fertility or inadequate breeding management (MacCallum 2004, Hogan *et al.* 2010c). Even in cases where mating has been successful, there is a high incidence of PY loss (V Nicolson 2012, personal communication). A number of factors have been reported to contribute to this phenomenon including: individual animal behavioural incompatibility issues (i.e. refusal to mate), poor body condition, diet, reversed social dominance (i.e. female dominant to male), inadequate breeding facilities (e.g. no blind tunnels), lack of space, infertility and failure to learn wombat-specific breeding behaviour due to hand-

rearing (Gaughwin *et al.* 1998, Jackson 2003, MacCallum 2004, Hogan *et al.* 2010b, 2010c). We must conclude that current understanding of Vombatidae reproductive behaviour, particularly those aspects related to mating success, oestrus prediction and identification are largely inadequate.

## Growth and development

### Pre-natal development and parturition

There are currently no studies on fertilisation biology in the Vombatidae. While Hughes & Green (1998) describe the pre-natal stages of embryonic development in the CW before hatching from the shell membrane, no definitive embryonic structures are present until mid-way through gestation. The wombat exhibits dual placental structures, which include a non-invasive but vascularised chorio-allantois and a yolk-sac; defined as a 'Type 3' placental structure according to Tyndale-Biscoe & Renfree (1987). With respect to parturition behaviour, a female CW was observed by Boer (1998) to be 'squatting' on her hindquarters with part of her weight supported by the forelimbs. Entire body tremors, 5–10 s in length, were observed and in-between these tremors the female rolled onto her side and grasped (with one forelimb) the area between the urogenital sinus and pouch opening. While a similar squatting position (Fig. 5) and body tremor was also noted in another CW (M Barnes 2012, personal communication, Dreamworld mammal keeper), this female did not roll onto her side, but instead bent forward and repeatedly tapped and/or kneaded her front paws on the ground. Barnes also noted that the female alternated between the squatting and bent forward positions, all the while vocalising, teeth grinding and body shaking. When squatting, the female made repeated thrusting movements with her hindlimbs, fully extending her hindlimbs for a few seconds (Fig. 5)



**Figure 5** Parturition postures observed in a captive common wombat (*Vombatus ursinus*). Female is 'squatting' and making repeated thrusting movement with the hindlimbs, fully spreading them and holding them extended for a few seconds (Barnes, unpublished data).

and then releasing muscle tension again. This behaviour went on repeatedly for over an hour. While the birth of the neonate (~2 g) was not witnessed by Barnes, Boer (1998) noted that following parturition, the neonate (<10 min) crawled its way from the urogenital opening to the pouch and attached to one of the teats without any maternal assistance.

### Pouch development

Repeated wild female capture is impractical, so as a consequence, wombat PY growth and development charts have been derived from captive-bred and hand-reared animals only. PY growth curves have been established based on mass and total body length for CWs and SHNWs, and mass and head length for SHNWs (Gaughwin 1982, Boer 1998, Taggart *et al.* 2007, Triggs 2009). No data on growth and development of PY are currently available for the NHNW. Figure 6 shows the variation between CW and SHNW PY mass growth curves (see review by Jackson (2003)). The growth rate of wombat PY is linear between birth and Day 310, after which growth slows and is best described by a polynomial equation (Taggart *et al.* 2007). Wild PY cannot be accurately aged using current captive growth curves due to the intrinsic differences between captive vs wild conditions; the mass of wild wombats varies as much as 40% between seasons and body length is also highly variable between individuals (Taggart *et al.* 2007, Triggs 2009). Wild PY age is best approximated using physical characteristics (Table 4). There is limited information available on wombat PY development (Table 4) with studies composed of incomplete data based on 1–2 individuals only (e.g. McLroy 1973, Crowcroft & Soderlund 1977, Gaughwin 1982, Presidente 1982). There appears to be a different rate of development between CW and SHNW PY with most physical characteristics in the SHNW developing earlier (Table 4); SHNW ears unfold at 30–60 days, whereas CW ears do not unfold before Day 90. Other aspects of development such as first release of the teat (135–140 vs 150–206 days), permanent pouch emergence

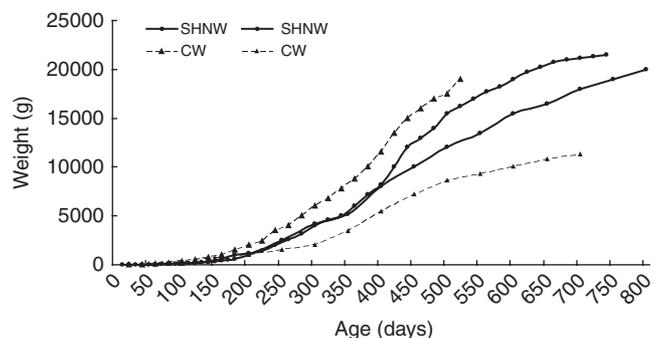
(235–270 vs 240–305 days) and weaning (340–365 vs 345–450 days) also occur earlier in the SHNW (Table 4). It has been suggested that climatic and/or dietary limitations on lactation and growth are less severe in the CW, enabling PY development to proceed at a slower pace (Taggart *et al.* 2007).

### Artificial reproductive technology

Artificial reproductive technology (ART) has considerable potential for improving the propagation and genetic diversity of wombat populations and has already been explored to a limited extent in the Vombatidae (e.g. sperm cryopreservation; AI; cross-fostering; ovarian hyperstimulation; ovarian tissue xenografting). Despite these advances, practical methods for manipulating wombat reproduction for the purposes of obtaining research material or for artificial breeding are not yet available. One of the major reasons for this is that the precise timing of oestrus and ovulation is not well documented, nor are the hormonal changes associated with these events.

### Semen collection and cryopreservation

Wombat seminal characteristics have been recorded in studies focusing on the monitoring of male fertility and on the development of electro-ejaculation (EJ) and semen cryopreservation techniques (Table 5). EJ techniques in the CW and SHNW were first described by Taggart *et al.* (1998) and have changed little since. In brief, the male wombat is anaesthetised before a lubricated multipolar rectal probe, with three longitudinally orientated strip electrodes inserted into the rectum. Semen is collected using an electrical stimulation unit and an EJ sequence (three series of 30 s of stimuli with 3–5 min rest periods between each series (Taggart *et al.* 1998) or multiple series of 3 s of stimuli for



**Figure 6** Pouch young growth curves (in body weight) for the common (CW; *Vombatus ursinus*) and southern hairy-nosed (SHNW; *Lasiorhinus latifrons*) wombat. Closed circles represent data from Taggart *et al.* (2007). Open circles and closed triangles represent data from Jackson (2003). Open triangles represent data from Boer (1998).

**Table 4** Pouch young development (in days) in wombats: a summary of published information.

Developing features	CW (Green & Rainbird 1987)	CW (Presidente 1982)	CW (Boer 1998)	CW (Triggs 2009)	SHNW (Gaughwin 1982)	SHNW (Taggart <i>et al.</i> 2007)
Vocalisations present					~176	~150
Incisors present	~112		~150	~120	90–120	99–107
Eyes						
Closed	<140	<105	<120	<120	<120	<120
Lashes			98–105	~90		~110
Open	~140	~105	120–155	>120	>120	120–145
Ears						
Fused			<110	<90		<30
Unfolding			110–120	~90	~30	30–60
Body fur						
None	<140	<180	<155	<150	<120	<143
Fine	140–196	180–210	155–261	150–180		
Thick	>196	>210	>261	>210	180–210	143–210
Teat					>210	>210
Lips fused	<84		<135	<150		<80
Lip vibrissae	~55–60		90–120		90–120	
Lips free	~84		~135	~150		80–92
On/off teat	~168		~206	~150		135–140
Pouch status						
In	<168	<255	<255	<180	<180	<160
In and out	168–224	255–285	255–270	210–240	180–210	160–210
Out	~280	>285	300–305	240–300	~270	235–265
Weaned	~350	~345	360–450	360–450	~360	340–365

CW, common wombat (*Vombatus ursinus*); SHNW, southern hairy-nosed wombat (*Lasiorhinus latifrons*).

a total period of 1–2 min (MacCallum & Johnston 2005)). Spermic ejaculates are recovered following stimulation with between 13 and 30 mA in the CW and 10 and 60 mA in the SHNW, with the percentage of successful semen collections ranging from 49 to 91% (Taggart *et al.* 1998, 2005, MacCallum 2004, Johnston *et al.* 2006). It is inferred in current literature that CWs are easier to EJ than SHNWs (Taggart *et al.* 2005, Johnston *et al.* 2006,

Hogan *et al.* 2010a) and that a better, SHNW-specific rectal probe should be designed in order to obtain a higher frequency of successful EJ attempts in this species. Ejaculates of both species typically have a high percentage of motile sperm (Table 5). MacCallum & Johnston (2005) have shown that wombat spermatozoa can also be recovered from post-mortem specimens and stored for upwards of 3 days in the intact cauda

**Table 5** Wombat seminal characteristics: a summary of published information.

Reference	Species	Ejaculate volume (ml)	Total sperm number ( $10^6$ /ml)	Percentage of motile spermatozoa	Sperm motility rating	Percentage live
Taggart <i>et al.</i> (1998) (ejaculate)	SHNW (wild)	≤7.5	9.0±6.2	90–95 (PF) 55–90 (PT)	4–5 (PF) 2–4 (PT)	
	CW (wild)	≤7.5	7.6±1.6	90–95 (PF) 70–90 (PT) 45 (PT)	5 (PF) 5 (PT)	
Bicknell <i>et al.</i> (2001) (epididymal)	CW (wild)					
MacCallum <i>et al.</i> (2001) (epididymal)	CW (wild)			60–62 (PF) 12–40 (PT)		79–81 (PF) 31–56 (PT)
MacCallum (2004) (ejaculate) (fast and slow freezing)	CW (captive)	3.9–7.8	3.8–78.5	83.4±7.3 (PF)	3.6±0.4 (PF)	94.3±2.6 (PF)
	CW (wild)		14–75	63.6±3.2 (PF)	3.6±0.3 (PF)	82.8±3.1 (PF)
	CW (fast)			10–35 (PT)	1.5–3.0 (PT)	30–60 (PT)
MacCallum & Johnston (2005) (epididymal)	CW (slow)			30–70 (PT)	3–4 (PT)	70–90 (PT)
	CW (wild)		29.3±9.9 (2–50)	20–80 (PF) 13–49 (PT)	1–4 (PF) 1–2 (PT)	30–90 (PF) 58–72 (PT)
Taggart <i>et al.</i> (2005) <sup>a</sup> (ejaculate)	SHNW (wild)	<1.0 (Jan)	2.7±4.4 (Jan)	58.3±30.5 (Jan)		
		3–9 (Sep)	34.0±31.5 (Sep)	89.8±10.8 (Sep)	4.5±0.5 (Sep)	
		<1.0 (Nov)	4.0±4.7 (Nov)	64.8±6.9 (Nov)	2.3±1.3 (Nov)	
Johnston <i>et al.</i> (2006) <sup>b</sup> (ejaculate)	CW (wild)			8–84 (PT)		

PF, pre-freeze; PT, post-thaw; CW, common wombat (*Vombatus ursinus*); SHNW, southern hairy-nosed wombat (*Lasiorhinus latifrons*); NHNW, northern hairy-nosed wombat (*L. krefftii*).

<sup>a</sup>Sperm motility index: 104.3±103.8 (Jan), 324.3±24.1 (Sep), 123.2±57.8 (Nov). <sup>b</sup>Percentage plasma membrane intact (0–2 h) 34–98 PT, percentage intact heads (0–2 h) 55–91 PT.

epididymides if the tissue is stored at 4 °C in a TCF (3.0 g Tris buffer, 1.7 g citric acid, 1.25 g fructose) buffer.

There have been numerous successful attempts to cryopreserve both cauda epididymal and ejaculated wombat spermatozoa (Table 5). Interestingly, wombat spermatozoa (like those of most other marsupials) do not appear to exhibit susceptibility to cold shock (Miller *et al.* 2004). As a result, wombat sperm cryopreservation diluents do not necessarily require the addition of egg yolk, but they do require a high concentration of glycerol in order to obtain adequate post-thaw survival. The cryopreservation success of wombat sperm is remarkable, with post-thaw motilities of  $\geq 80\%$  being regularly achieved in both species (Table 5). The most common way of cryopreserving wombat semen is in straws that are frozen in liquid nitrogen, but MacCallum & Johnston (2005) have also been successful at using a pelleting technique on dry ice. A higher degree of post-thaw survival is achieved when spermatozoa are frozen using a slow ( $-6$  °C/min to  $100$  °C in a programmable freezer) rather than fast (3 cm above liquid nitrogen for 30 min before being plunged into liquid nitrogen) rate of freezing (MacCallum 2004, Johnston *et al.* 2006). The main cryoprotectant medium used is a TCF buffer with egg yolk (15%) and glycerol (8 or 14%). Other cryoprotectants used include Krebs–Henseleit–Ringer base (17% glycerol), Triladyl (7% glycerol), 0.2 M trehalose (8% glycerol) and Tris–citrate–glucose buffer with 7.5% egg yolk and 14% glycerol (Taggart *et al.* 1998, Bicknell *et al.* 2001, MacCallum *et al.* 2001, Johnston *et al.* 2006). While cryobiological studies have examined the physio-chemical tolerances and biophysical properties of vombatid spermatozoa (MacCallum 2004, Miller *et al.* 2004, Johnston *et al.* 2006), the fertility of freshly collected or cryopreserved wombat sperm has yet to be tested.

### Artificial insemination

Successful AI protocols have only been developed in two marsupials: the koala and tammar wallaby. Contributing factors to the success of AI in the koala is that oestrus is easily detected and that ovulation can be induced (Johnston *et al.* 2003). In the tammar wallaby, birth and *post-partum* oestrus can be tightly synchronised by the removal of PY and ovulation is quite predictable, thus facilitating successful AI (Paris *et al.* 2005). As there are no reliable methods of oestrus detection, induction or synchronisation in the Vombatidae, the development of a successful AI protocol in wombats has been severely hindered. Druery (2004) attempted to determine the optimal timing for insemination in wombats by testing whether laparoscopy or transabdominal ultrasonography could be used to detect the presence of the pre-ovulatory follicle; these techniques were unsuccessful due to the opaqueness of the ovarian bursa (which obscured the ovary). Given the structural similarity of koala and

wombat reproductive anatomy, it is very likely that the methodology used for koala AI would be suitable for wombats. Recently (2012), Australian Animals Care and Education, Inc. (AACE; Mount Larcom, Queensland, Australia) in a joint research project with The University of Queensland (Gatton, Queensland, Australia) conducted a preliminary AI in two SHNWs using the same catheter design used for the koala (Johnston *et al.* 2003), but using frozen–thawed wombat caudal epididymal spermatozoa with a post-thaw motility of  $\sim 80\%$ . Using a vasectomised teaser male to detect oestrus in the female, two females were inseminated but no offspring were produced. AI experiments will continue into 2013 but using fresh EJ semen and close observation of oestrus using video surveillance. The AACE facility has over 20 wombats in its care and this large number will allow for more controlled experiments. Some of these females have recently been trained to urinate on demand so that samples can be analysed for hormone metabolites; this will be particularly useful as oestradiol metabolite measurement from SHNW faeces has not been instructive for monitoring oestrous cycle activity (Hogan *et al.* 2010b).

### PY removal and cross-fostering

Cross-fostering is an ethically challenging conservation technique. While it has the capacity to rapidly accelerate breeding and recruitment, the proliferation of a small captive group of animals can quickly lead to increased levels of inbreeding and/or over-representation of the genetics of a particular individual. This technique also poses a significant ethical issue, as it requires that the progeny of the non-threatened species to be killed, allowing the mother to raise the endangered young. Previous successes in other endangered marsupials (see review by Taggart *et al.* (2010)) and the poor breeding/recruitment of the NHNW merit the future development of PY isolation and cross-fostering techniques in the Vombatidae. Some of the ethical concerns associated with this technology could be reduced if oestrus synchronisation procedures, that allow the timed transfer of NHNW PY to CW or SHNW foster mothers at the appropriate stage of the reproductive cycle, are successfully developed. Nevertheless, it would still be a very brave animal manager who would make the decision to transfer a NHNW PY to a foster mother, in preference to allowing it to develop naturally.

Initially, Finlayson *et al.* (2007a) demonstrated the ability of female SHNWs to produce additional offspring in the same breeding season if a PY was lost or removed at an early stage of development. Thereafter, Finlayson *et al.* (2007b) demonstrated successful PY isolation and intra-species fostering in the SHNW. PY ranging in age from 16 to 146 days were isolated from the pouch (8-h at 23 °C and 100% RH) and successfully fostered between adult females. Females recaptured 4–11 months later

were still carrying the fostered PY or showed evidence of late lactation on the same teat that the fostered PY was originally attached to. Clearly, accelerated breeding through PY transfer is theoretically possible but further knowledge, leading to reliable captive husbandry and manipulation of the oestrous cycle, first needs to be established.

### **Ovarian hyperstimulation**

Druery *et al.* (2005) and West *et al.* (2007) have shown that ovulation occurs 25–26 and 36–45 h after LH administration in hormonally pre-treated CWs and SHNWs respectively. Improved ovulations rates and oocyte yields have also been achieved in both species following hormonal stimulation (West *et al.* 2003, 2007, MacDonald *et al.* 2006, Druery *et al.* 2007). In general, multiple administrations of FSH are required to stimulate follicular maturation, whilst a single LH injection is then required to induce ovulation of the recruited follicles. However, reproductive status before hormonal treatment does appear to affect stimulation outcomes, e.g. a higher production of large follicles and recovery of metaphase II (MII) oocytes occur when treatment is commenced early in the follicular phase of the natural oestrous cycle (Druery *et al.* 2007). Additionally, MII oocytes have only been collected from cycling adults or adults following PY removal (West *et al.* 2007). Only a low percentage (~14%) of oocytes have been reported to complete maturation to the MII stage (most are MI) following hormonal stimulation (Druery *et al.* 2007, West *et al.* 2007) indicating that current hormone dose rates and/or recovery times are not optimal. A major drawback of ovarian hyperstimulation in wombats is that ovary harvesting from cull specimens is presently required for the confirmation of stimulation effects. Druery *et al.* (2005) found that ultrasonographic imaging could not be used to confirm ovulation or the number of follicles in stimulated ovaries, while West *et al.* (2007) discovered that wombat oocyte recovery via laparoscopy was impractical.

### **Oocyte maturation**

*In vitro* maturation (IVM) and ICSI on CW oocytes have been attempted; it appears that immature oocytes generated either naturally or by superstimulation can be matured in culture. Cleary *et al.* (2003b) used 20 nM HEPES-buffered EMEM handling medium to culture oocytes and after 60 h, 34% matured to the MII stage. Similarly, 16% of oocytes cultured in TCM-199 medium matured to MII as indicated by the presence of a polar body (West 2002). The low maturation rates indicate that culture conditions will need to be further refined in order to optimise IVM. Maturation rates were greater in oocytes collected from follicles > 2 mm in size but were

not affected by the presence of cumulus cells (Cleary *et al.* 2003b). Future studies should investigate follicular size and the ability of resultant oocytes to mature *in vitro*. Oocyte IVF has been demonstrated in the CW, with several MII oocytes cleaving to the 2, 6 or 8 cell stage, 36–48 h following ICSI (West 2002, West *et al.* 2007); however, most of the injected oocytes (89%) degenerated following ICSI and culture. Failure of ICSI to fertilise MII oocytes has been attributed to factors such as oocyte viability, sperm preparation, the ICSI protocol and subsequent culture conditions (West 2002). There is still much to learn before IVM and IVF becomes a reality in the propagation of wombats and in light of both techniques being highly invasive, it is our opinion that the current focus of assisted reproductive technology needs to be on the development of ovulation induction, AI and PY transfer. The later techniques are ideally suited to the natural reproductive biology of the wombat, whereas the more invasive studies can be used for the purposes of gamete recovery from post-mortem animals or a better understanding of wombat fertilisation biology.

### **Ovarian tissue xenografting**

Xenografting of ovarian tissue is an advanced ART that can be used to allow growth and recovery of oocytes from cryopreserved ovarian tissue of females that die or are unable to reproduce naturally. In the initial xenografting study (Wolvecamp *et al.* 2001), CW ovarian tissue was grafted to the kidney capsule of immunocompromised rats and the follicles are allowed to grow to the preantral stage; the nude rat model was unable to support follicle development past the preantral stage (Wolvecamp *et al.* 2001). Cleary *et al.* (2003a) then investigated whether an alternative recipient would allow further follicular development and found that xenografting of CW ovarian tissue to immunocompromised mice resulted in follicular development to the antral stage. Immature oocytes were recovered from these antral follicles but failed to develop to MII after IVM (Cleary *et al.* 2003a). Graft recovery rates and follicular development were higher in ovarian tissue grafted under the kidney capsule, compared with grafts to subcutaneous sites, possibly because of more rapid revascularisation (Cleary *et al.* 2003a). Later, Cleary *et al.* (2004) showed that: ovarian tissue can develop to the antral stage when xenografted to male recipient mice; removal of the female recipient's ovaries accelerated follicle development and that no follicular growth occurs in xenografted ovarian tissue from PY or subadult CW females. As no oocytes were mature at the time of collection or after a period of IVM, it still remains unclear which conditions are best suited to generating viable oocytes from wombat ovarian xenografts (Cleary *et al.* 2004).

## Future directions

Future research directions for the development of ART in wombats should initially focus on understanding environmental, social, physical and nutritional influences on mating, fertility and reproductive success. Identification of the requirements vital to the successful breeding of wombats in captivity is of the utmost importance. Until PY can be produced on a regular basis (in captivity) there will be limited opportunity to further investigate topics like fertilisation, embryo development, parturition and cross-fostering. In addition, natural rather than artificial breeding is always preferable, as this ensures that wombats learn and exhibit normal species-specific sexual and parental behaviours. Obtaining a self-sustaining captive wombat population has proven difficult to date and as such knowledge of the physiology, endocrinology and behaviour of wombat reproductive events needs to be rapidly improved. Due to the intrinsic limitations associated with conducting studies within zoological institutions (e.g. low animal numbers, differing husbandry and management, unavoidable anthropomorphic impacts on behaviour, etc.) we also suggest the need for more dedicated 'wombat breeding and research centres', like that currently in development at AACE. Once sufficient animals and captive facilities are available, there will no doubt be a rapid acceleration in wombat breeding success.

While there has been a reduction in wombat population numbers, enough to prompt the Australian government to set in place and enforce legislation to actively protect this taxon in the wild, there are still a number of small CW and SHNW meta-populations that are regularly culled under mitigation permits (in areas where these animals impact agricultural practices or cause structural damage to buildings; [Temby \(1993\)](#)). While unpleasant, these mitigation programmes currently provide unique opportunities to investigate the reproductive anatomy and physiology of wombats that would not otherwise be possible; thus every effort must be made to use this extremely valuable reproductive material. Information gained could then be fed back into improving captive breeding programmes or used to develop and refine ART for the critically endangered NHNW.

Only after we can reliably and repeatedly breed wombats in captivity should we then switch our research emphasis to the development of more advanced forms of ART. Initially, research should centre on the development of AI and PY transfer techniques, as the natural biology of the animals (e.g. exceptional post-thaw sperm motilities and production of a second PY following removal of the first) can be capitalised upon and these techniques are far less invasive. The ability to artificially manipulate and/or enhance wombat reproduction will have significant applications, the most important being

aiding the recovery of the critically endangered NHNW. Despite an ongoing recovery programme for the NHNW (over the last two decades) it has been suggested that natural breeding, even in conjunction with habitat protection and restoration, may not be able to guarantee the survival of this endangered marsupial ([Horsup 2004](#)). While we hope that is not the case, it is important at the very least, to be proactive and develop ART as these techniques may, in the future, be the only way to protect the NHNW gene pool from further loss and at the same time help to propagate a sustainable captive population from which individuals could be reintroduced back into the wild.

Previous central QLD NHNW population monitoring has revealed that this population: i) is highly skewed towards males with only 35 females (and as few as 25 breeding) being present in the population ([Horsup 2004](#)); and ii) during years of average-to-above rainfall has 50–75% of adult females breeding ([Crossman \*et al.\* 1994](#)). Given that females can only produce one young every 2 years, a reasonable estimated annual recruitment rate (during optimal conditions) for this population ( $n=138-160$ ) would be about 6–9. However, the NHNW occupies an arid habitat within QLD with a high drought frequency, and most females ( $\geq 75\%$ ) will fail to breed in years with low rainfall ([Crossman \*et al.\* 1994](#)). During low-rainfall conditions, the annual recruitment rate for this population would mostly probably be 0–3. It is here that we see the case for enhancing the breeding success of the NHNW and development of ART is a vital approach towards providing NHNW population support.

## Declaration of interest

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

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