The novel reproductive biology of the female flying-fox and its implications for the successful development of an artificial insemination programme

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ABSTRACT

Flying-fox species worldwide are under threat of extinction. Artificial insemination (AI) has the potential to play a primary role in the conservation of endangered flying-foxes, through the genetic and reproductive management of captive colonies. Semen from surviving wild populations, or from separate captive colonies, can be utilised to maintain genetic vigour, thus preventing in-breeding in potential seed populations that can then be returned to restored habitat. The development of AI technology in flying-foxes has been hampered by the atypical reproductive biology of female Megachiroptera. Pteropids have a duplex uterus, with separate cervices, and a well-defined ovarian vascular complex that provides a counter-current exchange system between the ovary and ipsilateral uterine horn. This arrangement reduces systemic circulation of steroid reproductive hormones and makes it difficult to accurately characterise the endocrinology of the oestrous cycle; it is also consistent with the apparent lack of overt behavioural oestrus in these species. Low concentrations of peripheral oestradiol also mean that vaginal cytology is not a strong correlate of reproductive status. If AI is to be utilised as a conservation strategy in flying-foxes, it is vital that an accurate method of oestrus detection or ovulation induction be established. The integrated examination of plasma hormones, behaviour and vaginal cytology, following direct hormonal stimulation of folliculogenesis in the ovaries, may improve the signal to noise ratio in this subtle physiological system. Such improved sensitivity may make it possible to develop an accurate method of oestrus detection. Combined with the continuing development of the remaining steps in AI, this will ensure the progress of establishing an AI protocol in flying-fox species.

Key words: *Pteropus*, fruit bat, oestrus detection, progesterone, spermatozoa

Introduction

Many Pteropodidae species worldwide are under threat of extinction. Of the 65 species of *Pteropus* identified in the IUCN Red List, 54% were listed as threatened or extinct (IUCN 2009). Insufficient data were available to assess the status of 15%. The low reproductive rate of flying-foxes *Pteropus* species is a contributing factor to their demise. Females generally begin to breed during their second season, giving birth to only a single young each year (Hall and Richards 2000). If perturbed, their limited reproductive capacity makes them particularly susceptible to dramatic population losses from which they may take several years to recover, if at all (Mickleburgh *et al.* 1992). The removal/harvest of flying-fox food and roost trees for human development or agriculture (Fujita and Tuttle 1991), together with the threat of drought, cyclones, culling and over-hunting is making it increasingly difficult for some flying-fox species to naturally restore their numbers (Cheke and Dahl 1981; Craig *et al.* 1994; Brooke and Tschapka 2002). The limited distribution and abundance of island species also makes them prime candidates for extinction.

Of the seven Australian flying-fox species, the Dusky Flying-fox *P. brunneus* is reported to be extinct (Hall and Richards 2000) and both the Grey-headed Flying-fox *P. poliocephalus* and the Spectacled Flying-fox *P. conspicillatus* are currently listed as vulnerable (DEWR 2007). The important ecological role of flying-foxes as long-range pollinators and seed dispersers is often overlooked and/or ignored during times of human/flying-fox conflict, particularly surrounding urbanised camps and fruit orchards. Unfortunately, a broad range of ecosystems are likely to suffer if there is a reduction in flying-fox population numbers (McConkey and Drake 2006) so that the conservation of these species is of fundamental importance. Following the listing of *P. poliocephalus* as a threatened species in 2001, a forum was held to discuss the management of this species in New South Wales with the aim of developing a species management plan to ensure their conservation (Eby and Lunney 2002). Increased research on fundamental biology of flying-foxes was one of the recommendations that arose out of that forum (Recommendation 5, O'Brien and Fisher 2002), and the present work contributes to this by exploring the reproductive mechanisms of *Pteropus* species.

Traditionally, the conservation of endangered species has involved *in situ* conservation, such as habitat protection and restoration, which are often supported by

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public awareness campaigns. Another conservation strategy is the establishment of *ex situ* conservation programmes, such as the development of captive breeding colonies in zoos and wildlife sanctuaries (Mickleburgh and Carroll 1994). Captive colonies can serve as important insurance reservoirs of genetic diversity and in some cases may provide protection from entire extinction as a result of stochastic events or human induced catastrophes. Several captive Pteropid colonies have already been established at zoological institutions around the globe. The most famous captive colony is that of Rodrigues Fruit Bat *P. rodricensis*. This was first established in 1976 after the species was described in 1974 as possibly the world's rarest bat with an estimated wild population of only 75-80 individuals (Mickleburgh and Carroll 1994). In conjunction with the Mauritian government, Jersey Wildlife Preservation Trust initiated the development of a captive breeding programme. *Pteropus rodricensis* breed well in captivity, however, due to the polyandrous mating system there is a level of uncertainty surrounding paternity of the offspring. This uncertainty places constraints on the genetic management of the captive colony. Currently, the only method to ensure known paternity is to manipulate population dynamics and house this gregarious species in unnatural social groups. For many zoological institutions this would prove virtually impossible due to cost and space restrictions associated with additional housing requirements. Therefore, the benefits of *ex situ* Pteropid conservation would be further enhanced through the use of assisted breeding technologies, such as artificial insemination (AI) and gamete preservation, which would enable the precise genetic and reproductive management of these captive populations.

Artificial Insemination: A reproductive tool for conservation

Artificial insemination is a newly emerging field within captive wildlife conservation but is a tool that provides great promise for the enhancement of species conservation. The development of AI procedures also enables semen collected from surviving wild populations to be utilized to improve heterozygosity within captive populations whilst ensuring donor males still remain in the wild. In addition, it is less expensive and less stressful (to the animal) to globally transport preserved spermatozoa than individual animals (Wildt and Wemmer 1999). Gamete preservation also ensures that under-represented individuals are able to contribute their genes while at the same time enabling gametes and embryos from over-represented individuals to be frozen stored and 'cryofiled' for future use. The ability to cryopreserve gametes indefinitely can also guard against short generation intervals and catastrophic events (Wildt and Wemmer 1999) such as disease outbreaks, fire and cyclones that may decimate isolated wild flying-fox populations (Cheke and Dahl 1981). This point was brought home in 1991 when the island of Rodrigues in the Indian Ocean was devastated by a cyclone and the endemic population of *P. rodricensis*, which had increased to over 1000 individuals since 1976, fell dramatically to approximately 350 individuals (Mickleburgh and Carroll 1994).

The importance of captive flying-fox colonies for the development of an artificial insemination programme

A comprehensive understanding of basic reproductive processes is fundamental to the reproductive management of captive non-domestic species and in particular to the development of assisted breeding techniques (Wildt 1997). Male and female reproductive biology should be understood before AI can be successfully implemented. Therefore, the ability to assess an individual animal's reproductive capabilities can only be reliably achieved through an understanding of what is 'normal' (Wildt 1997). The establishment of captive populations of Pteropids can present opportunities for scientists to increase knowledge of the reproductive biology and physiology of these species, through the provision of a readily available source of animals. This enables repeated observations of known individuals over time, thus providing a clearer indication of 'normal' biological and physiological reproductive parameters (Wildt 1997). Reproductive researchers have previously utilized captive colonies of flying-foxes to improve knowledge of Pteropid reproduction. Some of this research included information on coital behaviour (O'Brien and Nankervis 1994), reproductive hormone levels in pregnant and non-pregnant females (Towers and Martin 1995) and seasonal changes in testicular size and plasma testosterone concentrations (McGuckin and Blackshaw 1991).

There are three major areas of development in establishing an AI programme irrespective of the species in question (Johnston and Holt 2001). These are: developing a method of semen collection and preservation; determining where to deposit the semen within the female's reproductive tract; and identifying the optimal time to inseminate the female. Successful preservation of flying-fox spermatozoa represents a significant challenge, particularly given the large and fragile nature of the Pteropid acrosome (de Jong *et al.* 2005). However, the 'when' and 'where' questions of artificial insemination in the flying-fox are equally challenging, given the anatomy and complicated physiology of the female flying-fox reproductive system.

Developing AI - step 1: semen collection and sperm preservation in the flying-fox

Successful semen collection and sperm preservation are fundamental to the establishment of an AI programme. In many domestic species natural semen ejaculates are collected by artificial vagina (stallion, bull, ram) or by

manual stimulation (dog). We recently reported on the collection of flying-fox semen using manual stimulation in a habituated male Black Flying-fox *P. alecto* (Melville *et al.* 2008). Unfortunately, the collection of natural ejaculates for preservation or artificial insemination is usually not possible in the case of wild species so that semen is typically collected by electro-ejaculation while the animal is under general anaesthesia. Electro-ejaculation involves the stimulation of the male reproductive tract to artificially induce the emission of an ejaculate (Watson 1990). In the flying-fox, a small probe (de Jong *et al.* 2005) is gently inserted into the rectum of an anaesthetised animal and a series of weak electrical stimulations applied. Our research group has routinely used electro-ejaculation to successfully collect semen samples from three flying-fox species (*P. alecto, P. poliocephalus* and Little Red Flying-foxes *P. scapulatus*) with the animals showing no adverse reactions to the procedure (de Jong *et al.* 2005; Melville 2006).

While there is a well-defined seasonal pattern of change in flying-fox testicular size and testosterone secretion (McGuckin and Blackshaw 1991; O'Brien 1993), the effect of seasonality on flying-fox semen quality is currently under investigation (Melville unpublished observations). Interestingly, seminal vesicle secretions typically found in ejaculates during the mating season are usually absent from ejaculates collected at other times of the year (*P. scapulatus* O'Brien 1997; de Jong *et al.* 2005; *P. poliocephalus* Melville 2006) and their relationship to sperm fertility requires further investigation.

The flying-fox spermatozoon possesses a large and fragile acrosome that is particularly sensitive to *in vitro* manipulation and preservation techniques (de Jong *et al.* 2005; Melville 2006). Figure 1 shows some of the morphological damage caused to the acrosome following chilling or cryopreservation. The anterior projection of the acrosome is likely to make it vulnerable to external stressors, particularly those that are osmotic or mechanical in nature. Acrosomal damage can also be caused by the phenomenon of cold shock, a problem which can occur when cells are rapidly cooled to just above 0°C during preservation procedures (Holt 2000). Successful preservation of flying-fox spermatozoa will require a detailed understanding of the factors that initiate or mediate acrosomal damage or loss so that these processes can be minimised during manipulation and preservation. Premature damage to the acrosome results in a reduction of fertilization capability and is, therefore, a major concern for the future development of flying-fox AI based on preserved spermatozoa.

Developing AI - step 2: female Pteropid reproductive anatomy and determining insemination location

Despite variations between the anatomies of the reproductive tract amongst female Pteropids, all species that have been examined possess a primitive duplex uterus-cervix complex (Hood 1989). The oviduct of *Pteropus* spp. characteristically wraps around the lateral and cranial aspects of the spherical to ellipsoid-shaped ovary, which histologically resembles that of other eutherian mammals. In many microchiropteran species the reproductive tract is anatomically or physiologically asymmetrical (Wimsatt 1979) but in the Pteropids both ovaries function (Marshall 1947). Flying-fox ovaries are contained within an ovarian bursa making visualisation of ovarian activity difficult. In fact, some standard techniques used to time AI, such as laparoscopy, are technically not feasible given the ovarian bursa and the small size of the animal.

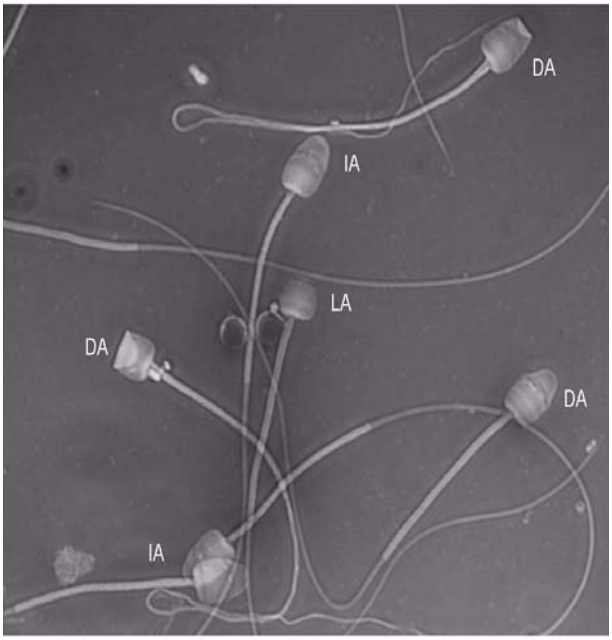

Figure 1. Morphological damage caused to the acrosome of *P. poliocephalus* spermatozoa following chilling. DA – damaged acrosome, IA – Intact acrosome, LA – lost acrosome.

Vascular perfusion and drainage of the ovary and adjacent uterus are highly modified in Pteropids. There is a counter-current vasculature exchange system between each ovary and ipsilateral horn of the uterus (Pow and Martin 1994). Pow and Martin (1994) investigated the relationship between the ovarian-uterine blood supply and the endometrium ipsilateral to the ovulating ovary. They found that the ovarian artery coils extensively on the cranial side of the ovary before continuing caudally to the cranial tip of the uterus. Primordial follicles are restricted to the caudal pole of the ovary, which results in the internalised corpus luteum forming cranially in the ovary, close to the coil of the ovarian artery. This coil is completely enclosed by a venous sinus that drains the cranial pole of the ovary. The close proximity of the ovarian artery and vein in the vascular complex is thought to allow for a counter-current or cross-current transfer of ovarian steroids from ovarian vein to ovarian artery and on to the cranial tip of the ipsilateral uterine horn (Pow and Martin 1994; Figure 2). In this way reproductive steroids produced by the ovary reach high

concentrations at the ipsilateral uterine horn. This generates localised endometrial growth in the ipsilateral uterine horn. Systemic levels of reproductive steroids remain relatively unchanged, such that measurement of the peripheral concentrations provides little value in the assessment of reproductive status (Towers and Martin 1985a).

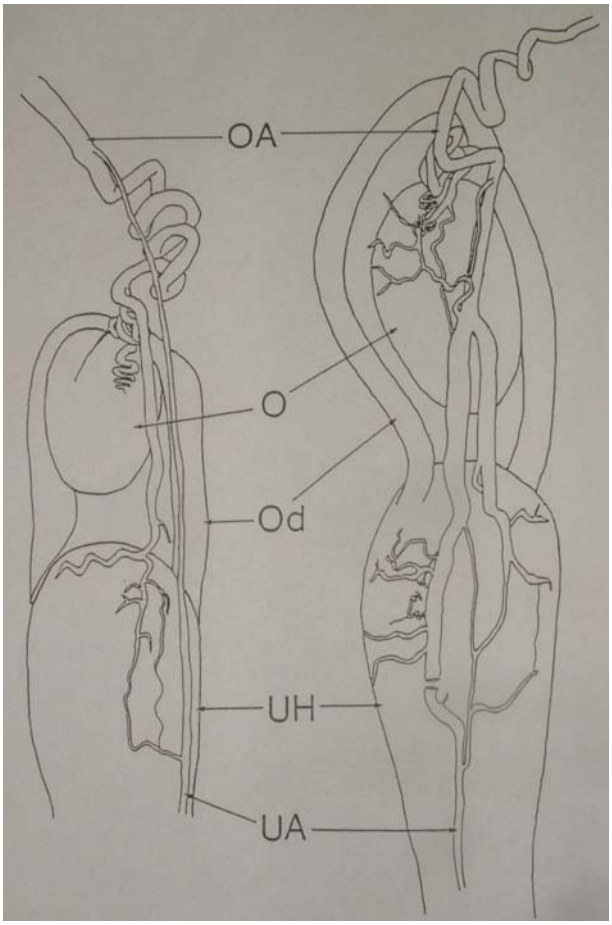

Figure 2. The dorsal aspect of vascular casts of the cranial tips of uteri and associated structures from two non-pregnant *P. scapulatus* females (Pow and Martin 1994). O – ovary, OA – ovarian artery, Od – oviduct, UA – uterine artery, UH – uterine horn.

The site of artificial insemination can vary between species. Sperm survivability within the female is limited and depositing semen into the correct location within the female's reproductive tract will maximise fertilization success (Ax *et al.* 2000). In the mare and cow, semen is artificially deposited directly into the uterine body, while in the ewe and sow, it is usually deposited into the cervix (Ax *et al.* 2000). In some instances when donor semen has high economic merit, surgical insemination directly into the oviduct or tip of the uterine horn may be used. However, given that Pteropids have a duplex uterus with separate cervices and ovulations occur from alternate ovaries, options for the site of semen deposition are currently limited to the vagina. Since semen is naturally deposited by the male into the vagina, this would appear to be the logical place to test first. Preliminary observations using coloured liquid deposited into the vagina of deceased female *P. alecto* have shown that the inseminate was able to pass through the cervices, uterine horns and reach the oviduct (Figure 3, located at the end of the paper). This provides a degree of hope that this method may be suitable for a live flying-fox. A similar method has been successfully employed in the koala with semen deposited into the urogenital sinus immediately adjacent to the openings of the lateral vaginae (Johnston *et al.* 2003).

Developing AI - step 3: female Pteropid reproductive physiology and the timing of insemination

Gonadotrophin releasing hormone (GnRH) secreted from the hypothalamus acts on the gonadotroph cells of the anterior pituitary to produce and release follicle stimulating hormone (FSH) and luteinizing hormone (LH). In the female, varying pulsatile secretion of FSH and LH stimulates folliculogenesis resulting in the increased development of the antral follicle. During follicular development, oestrogen is secreted from the granulosa cells of the developing ovarian follicle and there is a positive feedback of oestrogen on the release of LH. The developing follicle also secrets inhibin, which acts to suppress FSH secretion. Once a critical threshold of oestradiol is reached, at least in spontaneously ovulating species, a surge of GnRH is released from the hypothalamus resulting in a subsequent release of LH from the anterior pituitary. This surge of LH has a direct effect on the ovary resulting in the ovulation of the oocyte from the follicle. While the oocyte moves through the ovarian bursa and oviduct, LH continues to act on the remainder of the follicle.

Sexual behaviour and female receptivity in mammalian species are closely linked with increased levels of oestrogen. The luteal phase of the female's reproductive cycle begins after ovulation, oestrogen levels rapidly fall and the corpus luteum begins to form from the luteinization of the granulosa and thecal cells of the collapsed follicle. The cells that were once responsible for the production of oestrogen are transformed into luteal cells which start to secrete progesterone. Progesterone is responsible for preparing the female's reproductive tract for implantation of the embryo and for maintaining pregnancy. If fertilisation is not successful, the corpus luteum is not maintained. Once the progesterone concentration falls, the inhibitory effect of progesterone on the hypothalamus is removed and the oestrous cycle will begin again. On the other hand, if fertilization is successful then the corpus luteum will continue to produce progesterone for a longer period of time (species-dependent) before it begins to regress. In most domestic species, female reproductive status can be determined by simply collecting blood samples and monitoring these changes in hormone levels. Unfortunately, determination of the reproductive cycle in the female flying-fox has not proved quite as simple.

The ovarian-uterine vasculature complex of Pteropids is believed to have the effect of reducing the systemic circulation of steroid reproductive hormones, such as

progesterone and oestrogen produced from the ovaries (Pow and Martin 1994). However, systemic levels of these hormones were shown to be substantial during the latter half of pregnancy when the corpus luteum had regressed and the placenta assumed hormonal control of the pregnancy (Towers and Martin 1995). Given the lack of detectable changes in reproductive steroids in the peripheral circulation of the flying-fox (except during late pregnancy) it is extremely difficult to accurately characterise the endocrinology of the oestrous cycle or predict reproductive events. The lack of a detectable change in the level of peripheral oestrogen in the flying-fox is puzzling as oestrogen in other mammals has a major role in inducing a GnRH and LH surge for ovulation and is the primary hormone controlling behavioural oestrus. Not surprisingly, Martin and Towers (1985) reported a distinct lack of oestrus behaviour in flying-foxes which hinders the ability to determine the timing of ovulation in these species. Martin *et al.* (1995) reported that both intact and ovariectomized females tend to copulate repeatedly and over long time periods without any reference to oestrus; in addition, intact females will continue to copulate well into pregnancy (Puddicombe 1981; O'Brien 1997).

In most mammalian species, oestrus can be detected by monitoring changes in reproductive hormone concentrations and/or through resultant changes in vaginal cytology. Typically, the proportion of particular epithelial cell types in the vaginal mucosa change as the female comes into oestrus. An increase in cornified squamous epithelial cells of the vagina is normally associated with the female preparing for intromission by the male. However, the vaginal epithelium in flying-foxes remains cornified, resulting in an inability to use vaginal cytology to characterise the oestrous cycle (Kennedy 1992). Vaginal smears collected by Towers and Martin (1985a) from *P. poliocephalus* females did not show any significant changes in cell composition related to the female's reproductive status. However, in *P. scapulatus* changes in vaginal cytology were observed during late pregnancy when systemic levels of gonadal steroids are elevated (O'Brien 1997). Perhaps experiments in which oestrus can be artificially induced through hormonal stimulation might be useful in helping to document systemic changes in plasma oestrogen and changes in vaginal cytology associated with oestrus.

Kennedy (1992) has shown that high levels of circulating inhibin (a non-steroidal hormone) in the flying-fox are associated with the presence of a corpus luteum. Inhibin levels typically increase sharply and remain high until about half way through gestation at which time the corpus luteum regresses and the placenta takes over hormonal control (Kennedy 1992). After this point, placental progesterone can be measured. Consequently, measuring inhibin levels might at least indicate if an individual has produced a functional corpus luteum, signifying that ovulation has now occurred and pregnancy has begun. It is possible that plasma inhibin in Pteropids would be a better indicator of ovarian function than changes in oestradiol and progesterone (Kennedy 1992). In Australian flying-foxes, it is the presence of a preovulatory follicle which causes the ipsilateral uterine horn to exhibit hormonal stimulation; the contralateral horn does not respond to these changes (Martin *et al.* 1995). Progesterone, a hormone typically associated with ovarian function, was shown to persist at high levels in some ovariectomized females whereas inhibin levels became undetectable. This indicated that progesterone has a non-ovarian source whilst inhibin appears to be directly related to the presence of the ovary. Kennedy (1992) also determined that there was no significant correlation between inhibin and progesterone levels.

Future directions for establishing methods of oestrus detection in the flying-fox

The ability to detect oestrus is important for the timing of AI. One method of detecting oestrus is through behavioural observations. Females of most species (humans excluded) typically display what is termed as 'overt behavioural oestrus'. These behaviours may include: seeking out males, engaging in homosexual behaviour such as mounting other females, increased locomotion, standing for mounting and vocalisation. The frequency of these behaviours increases with elevated oestrogen levels. Some species, as is the case for humans, display what is termed as 'silent oestrus', where there are no apparent detectable behavioural clues indicating receptivity in the female.

An intensive study of the courtship behaviour of flying-foxes in the wild failed to identify changes in behaviour that would signal oestrus (Markus 2002), suggesting that perhaps flying-foxes display a silent oestrus. It may be that flying-fox females are always receptive and mating interaction is a result of male libido and sexual interest (Martin *et al.* 1995). Alternatively, female flying-foxes may give off subtle clues or pheromones, the olfaction of which is detected by the male. Female flying-foxes have been observed copulating with multiple males and often well into pregnancy (Puddicombe 1981; O'Brien 1997). This pattern of mating activity tends to suggest that receptivity in these species is not predictive of ovulation.

An alternative method routinely used in some AI programmes is to induce oestrus through the administration of reproductive hormones. This is typically done in cattle and sheep to synchronise female breeding groups. Hormonally induced oestrus in domestic animals usually involves the administration of progesterone slow release devices that are normally inserted into the vagina. These devices are removed after several days and the female is usually injected with a low dose of pregnant mare serum gonadotropin (PMSG) or combination of FSH/LH which stimulates follicular growth and ovulation (Ax *et al.* 2000).

Following on from the work of Martin, O'Brien, Towers, Pow and Kennedy, which was conducted at the University of Queensland during the late nineteen eighties/early nineties, our research group is currently trying to establish a method of reliable oestrus detection or ovulation induction in the flying-fox. By

understanding the relationship between displays of overt behavioural oestrus (if they exist) and the timing of ovulation in flying-foxes, we should be able to predict the best time to inseminate the female to ensure ovulation has occurred and thereby increase the possibility of fertilization. To ensure this is possible, an accurate method of oestrus detection needs to be established. Alternatively, if we are able to effectively and reliably pharmaceutically induce ovulation in flying-foxes it may not be totally essential that we detect behavioural oestrus.

Research conducted by Towers and Martin (1985b) showed that administration of PMSG can stimulate folliculogenesis in *P. scapulatus* with ovulation expected to occur around day four after injection. Unfortunately, the dose rate used by Towers and Martin (1985b) resulted in multiple ovulations in some cases. Recent studies have shown that multiple pregnancies in the flying-fox may present some risk to the welfare of the mother and the potential young (Fox *et al*. 2008). Consequently, oestrus induction protocols in the flying-fox should aim to result in a single ovulation. To reduce the possibility of superovulation, a more appropriate PMSG dose rate will first need to be established.

Conclusion

As a result of continued habitat loss and the inherent low reproductive rate of flying-foxes, Pteropid populations are in urgent need of conservation. Whilst not intended to replace traditional conservation approaches, such as public education, habitat protection and restoration, AI can provide important tools for the assessment of reproductive status and genetic management. Improvements to sperm preservation methods will ensure that the best quality spermatozoa are inseminated; however, determination of the site of insemination may prove more challenging due to the presence of dual cervices in the female flying-fox. Additionally, to successfully develop an AI programme in flying-foxes, it is essential that an accurate and reliable method of oestrus detection or ovulation induction be established. This will ensure that insemination occurs at the most appropriate time to optimise pregnancy success. An increase in the knowledge of both male and female flying-fox reproductive biology and the associated development of assisted breeding techniques will undoubtedly help conservation efforts in these species.

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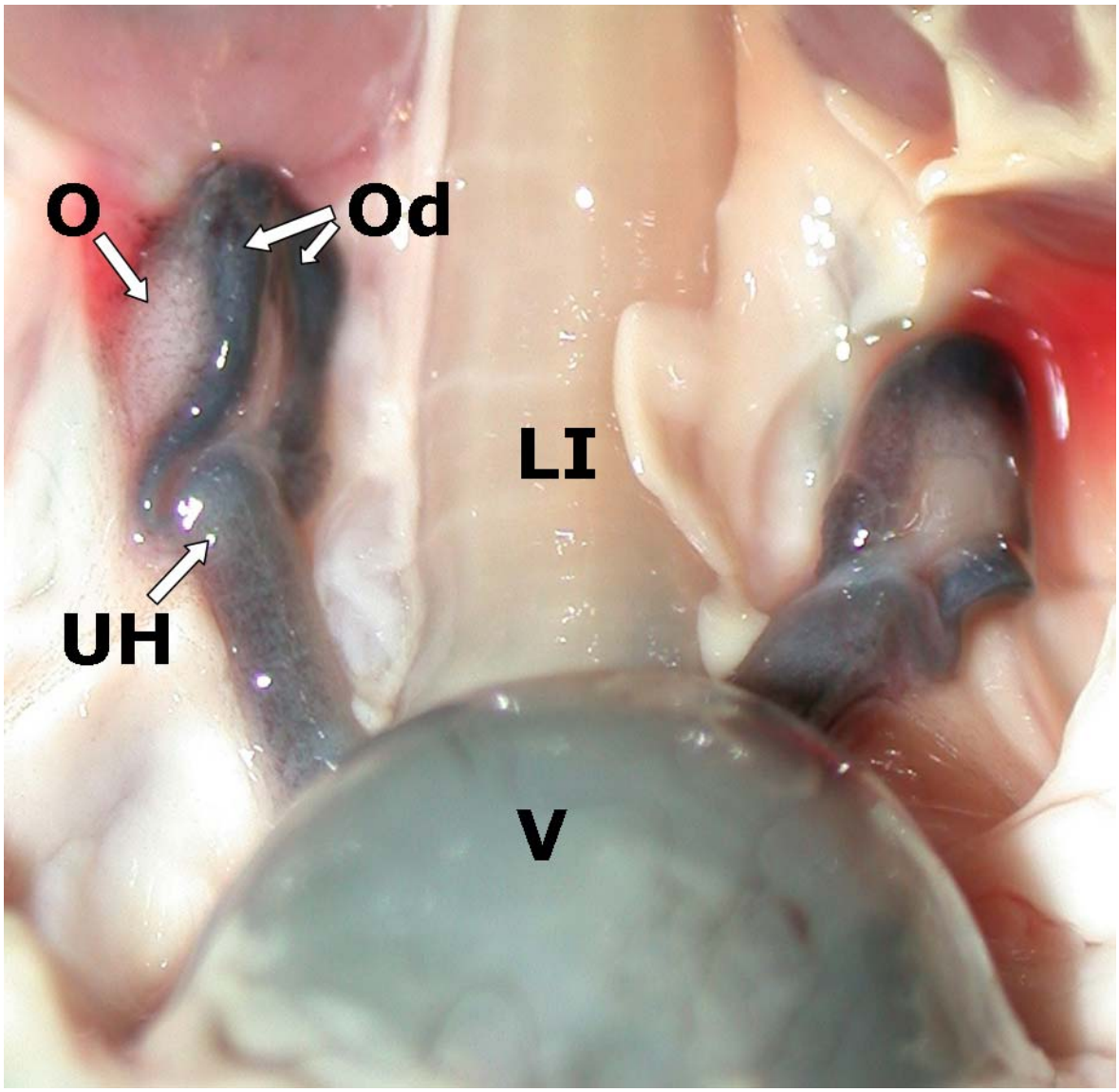

Figure 3. Preliminary observations, using coloured liquid, into the site of semen deposit in Pteropids. LI – large intestine, O – ovary, Od – oviduct, UH – uterine horn, V – vagina.