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First report on the colpocytology and serum steroid hormone levels of Giant otters *Pteronura brasiliensis*

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Considering the importance of tools for the reproductive monitoring and management of Giant otters *Pteronura brasiliensis* in the wild or in captivity, this study reports the relationship among the colpocytology, reproductive serum hormone (progesterone and oestradiol) levels and ovarian morphology of adult Giant otters. Blood and vaginal cytology samples were obtained from four adult females (two free-ranging and two in human care). The ovaries were collected from the captive females. The data were correlated to define the phase of the oestrous cycle of each animal. The results suggest the feasibility of using hormone analysis or colpocytology to aid in the reproductive monitoring of Giant otters in human care. In addition, these tools could be used to define the reproductive status of individual wild Giant otters during capture procedures.

Key-words: giant otter; monitoring; mustelid; oestradiol; progesterone; reproduction; vaginal cytology.

INTRODUCTION

The Giant otter *Pteronura brasiliensis* is an endemic semi-aquatic mustelid from South America (Duplaix *et al.*, 2015). The species is classified as Endangered on the International Union for Conservation of Nature (IUCN) Red List because of intensive hunting for the international pelt trade in the past, current conflicts with fishermen, and the destruction and degradation of its habitat (Rosas-Ribeiro *et al.*, 2012; Groenendijk *et al.*, 2015; Antunes *et al.*, 2016). Adult Giant otters in the Amazon region usually measure 1·5–1·8 m (‘snout–tail tip’ length) and have a mean weight of 22–26 kg, without significant differences in the size and weight of males and females (Rosas *et al.*, 2009; Duplaix *et al.*, 2015). Behavioural and anatomical observations suggest that males and females reach sexual maturity at around 2–3 years of age (Oliveira *et al.*, 2011; Groenendijk *et al.*, 2014; Bozzetti *et al.*, 2015). The Giant otter is a monogamous species and only the alpha couple reproduces in the group; however, some studies suggest the existence of reproductive activity in other group members (Ribas *et al.*, 2016). Wild Giant otters show reproductive seasonality; however, in zoological institutions the species has been shown to reproduce all year round (Hagenbeck & Wunnemann, 1992; Evangelista & Rosas, 2011; Bozzetti *et al.*, 2015; Metrione *et al.*, 2018).

Giant otters have been kept in zoological institutions in South America, North America, Europe and Asia, and some efforts have been made to breed this species over

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recent decades (Sykes-Gatz, 2005; Brandstätter, 2011; Metrione *et al.*, 2018). For breeding to be successful, knowledge of oestrous-cycle physiology, including hormone levels during each phase, is extremely important for monitoring reproductive individuals. The serum steroid hormone measurement is an excellent tool for reproductive monitoring because the serum levels of these hormones are directly correlated with ovarian activity of individuals.

Vaginal cytology, or colpocytology, is another low-cost tool used to monitor the oestrous cycle in various species, primarily in Domestic dogs *Canis familiaris*. The vaginal mucosa cytology alters in response to the levels of reproductive steroid hormones related to variations in ovarian tissue (presence of growing follicles or corpus luteum) (Schutte, 1967b; Post, 1985; Wright & Parry, 1989). In mustelids, oestrous cycle evaluation by colpocytology has only been reported for a few species of the *Mustela* genus (Williams *et al.*, 1992; Young *et al.*, 2001), and North American river otter *Lutra canadensis* and Neotropical otter *Lontra longicaudis* (Stenson, 1988; Nidasio & González, 2009). This study reports on the relationship among colpocytology, reproductive serum hormone (progesterone and oestradiol) levels and ovarian morphology in adult Giant otters.

MATERIALS AND METHODS

Blood and vaginal cytology samples were obtained from four adult Giant otters (Table 1). Two free-ranging females (F-1 and F-2) were captured and submitted to a surgical procedure for intraperitoneal transmitter implantation for a telemetry-monitoring project (Rosas *et al.*, 2015). Both females were caught in February 2012. F-1 was a female with transient behaviour and was observed copulating with a transient male. Female F-2 was the alpha female of her group and the only female in that group. She had a 4 month-old pup. The capture protocol was as previously described for the species (Silveira *et al.*, 2011), and carried out under ethical and governmental authorizations (SISBIO No. 27396-6, and INPA Animal Research Ethics Committee No. 006/2013). The other females (F-3 and F-4) were captive-reared animals that were chemically restrained for veterinary clinical examination because they had symptoms of health problems. At examination, F-3 was 12 years of age and F-4 was 18 years of age, and these females were examined in February 1999 and February 2016, respectively. It is purely coincidental that all handling procedures in this study occurred in February. During handling, a blood sample was collected from the jugular vein and the vaginal mucosal epithelium sample was collected using a swab from each female. Colpocytology was not performed on F-3 because the procedure had not been planned to be carried out at that time.

The day following examination F-3 died and F-4 died 6 days after handling; both deaths were the result of pre-existing illnesses. During necropsy, the ovaries were collected from these two females and fixed in 10% formaldehyde, making it possible to investigate ovarian morphology.

Vaginal smears were generated using cytological samples of the vaginal mucosa and stained using the rapid panoptic method. The slides were analysed under an optical microscope for qualitative evaluation of cell types according to Schutte (1967a).

Blood samples were centrifuged and serum was stored at −20°C until hormonal

ANIMAL	LENGTH (cm)	WEIGHT (kg)	ORIGIN
F-1	149	21·0	wild
F-2	161	22·2	wild
F-3	153	20·0	captivity
F-4	160	22·5	captivity

Table 1. Weight, length and origin of Giant otter *Pteronura brasiliensis* females evaluated in a study into colpocytology and serum steroid hormone levels.

analysis. Serum progesterone and oestradiol levels were evaluated using an enzyme immunoassay protocol previously validated for different animal species (Munro *et al.*, 1991; Graham *et al.*, 2001) and modified by Amaral *et al.* (2018). The CL425 and R0008 antibodies, for progesterone and oestradiol, respectively, and their respective conjugated hormones, were provided by the University of California, Davis (USA). Serial dilutions of pooled serum generated dose-response curves in parallel to serially diluted hormone standards for progesterone and oestradiol. Assay sensitivity was $0{\cdot}08\ \mathrm{ng\ ml^{-1}}$ for progesterone and $0{\cdot}17\ \mathrm{ng\ ml^{-1}}$ for oestradiol. Intra-assay coefficients were <9·8% for both assays.

The ovaries of deceased females (F-3 and F-4) were macroscopically evaluated (presence of antral follicles and corpus luteum). Hormonal, cytological and morphological results were compared and correlated.

RESULTS

The hormonal levels, cellular profile and ovarian morphology observed in the study are presented in Table 2.

DISCUSSION

The vaginal epithelial cells in Giant otters are morphologically similar to those described in dogs (Schutte, 1967a) and other mustelids (Stenson, 1988; Williams *et al.*, 1992; Young *et al.*, 2001; Nidasio & González, 2009).

In general, pro-oestrus in dogs was characterized by the predominance of intermediate and superficial cells, and this cellular characteristic was related to ovarian follicle growth and increasing oestradiol levels (Schutte, 1967b). Oestrus was predominated by keratinized superficial cells, whereas metoestrus and dioestrus were predominated by intermediate and parabasal cells, and a large number of leucocytes, which were related to corpus luteum formation and maturation, and progesterone production (Schutte, 1967b). During mid- and late dioestrus, an increase in parabasal cells had been noted, and neutrophils were typically absent. Anoestrus was characterized by few intermediate and parabasal cells (Schutte, 1967b).

An increase in keratinized superficial cells was detected in vaginal cytology from pro-oestrus to oestrus, and a predominance of intermediate and parabasal cells during anoestrus was also reported in different mustelids species (Stenson, 1988; Williams *et al.*, 1992; Nidasio & González, 2009). Nidasio & González (2009) also observed high levels of oestradiol in a female Neotropical otter showing oestrous behaviour.

Based on reports for other carnivores, including some mustelids, the observations made in the present study suggest that the wild females were at the beginning (F-1) and mid–late (F-2) dioestrus based on high levels of progesterone, low levels of oestradiol, and the predominance of parabasal and intermediate cells with presence of neutrophils in vaginal smear. According to Rosas *et al.* (2007) and Evangelista & Rosas (2011), wild Giant otters in the Amazon show reproductive seasonality with births mainly occurring during the periods of high and receding water (July–December). The gestation period in Giant otters is *c.* 66 days (Metrione *et al.*, 2018). Although delayed implantation has been previously reported for other mustelids (Fenelon *et al.*, 2017), the occurrence of delayed implantation in Giant otters is unknown. Thus, considering the date of capture of the Giant otters in this study, they were possibly showing the first cycles of the season, which seems to be corroborated by the copulation observed in female F-1.

The results from F-3 also suggest dioestrus. Although the species shows reproductive seasonality in the wild, it can reproduce throughout the year in captivity (Hagenbeck & Wunnemann, 1992; Metrione *et al.*, 2018), explaining the cyclicity observed. Additionally, the occurrence of

ANIMAL	PROGESTERONE (ng ml^{-1})	OESTRADIOL (ng ml^{-1})	COLPOCYTOLOGY	OVARIAN MORPHOLOGY
F-1	5·57	0·59	predominance of intermediate cells and presence of neutrophils	
F-2	1·41	< 0·17	few parabasal cells	
F-3	1·58	0·77		presence of small antral follicles and small corpus luteum
F-4	0·76	4·30	predominance of keratinized superficial cells and few anucleated keratinized cells	large antral follicle and small antral follicles

Table 2. Hormonal levels, colpocytological description and ovarian morphology of four Giant otter *Pteronura brasiliensis* females analysed in a study into colpocytology and serum steroid hormone levels.

pseudopregnancy has been reported in some individuals with sustained high progesterone levels (Metrione *et al.*, 2018). Behaviour suggestive of pseudopregnancy, including the development of mammary glands, has also been reported in wild and captive females (Rosas & de Mattos, 2003). However, there is no additional reproductive information from the females evaluated in this present study to indicate the progression of their oestrous cycles.

The F-4 female exhibited proestrus/oestrus as indicated by high oestradiol levels, the predominance of keratinized cells on the vaginal smear and the observation of antral follicles in the ovary. This female was the oldest subject evaluated in this study (18 years). Giant otters can live up to 20 years in zoological institutions (Oliveira *et al.*, 2007), and the existence of reproductive senescence in this species is unknown. However, Metrione *et al.* (2018) also observed reproductive activity of 18 year-old Giant otters in human care, supporting the results obtained here.

These data suggest the feasibility of using hormone analysis or colpocytology to aid in the reproductive monitoring of Giant otters in managed care. These tools could also be used to define an individual's reproductive status during the capture of wild Giant otters. Blood samples and vaginal smears are invasive procedures and to carry them out the animals must be restrained. However, in zoological collections the stress of such procedures could be minimized by using animal training to make it possible to collect samples without unfamiliar restraint (Sykes-Gatz, 2005).

Recently, faecal-hormone measurements have been used as a non-invasive tool for longitudinal reproductive monitoring of Giant otters (Metrione *et al.*, 2018). However, those authors were unable to report on some individualized results because of sample-collection limitations, such as mixed faeces from communal latrines used by Giant otters. Therefore, a combination of tools would be extremely useful for better reproductive monitoring and the development of effective breeding programmes.

In conclusion, this is the first report on serum steroid hormone levels colpocytology evaluation in *P. brasiliensis*, which can aid in the reproductive monitoring of Giant otters. Further studies are necessary to gain a better understanding of the hormonal profile of Giant otters during the oestrous cycle and its relationship to vaginal epithelial cell morphology.

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