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REPRODUCTIVE ACTIVITY AFTER INDUCED ANESTRUS USING ALTRENOGEST IN *Tursiops truncatus* FEMALES IN CAPTIVITY IN MARINE ENVIRONMENT

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ABSTRACT

Interest to reproduce Bottlenose dolphin (*Tursiops truncatus*) in captivity has increased due to the international restrictions for its commercialization and the risks and logistical difficulties for transporting specimens. Therefore, it has become important to study its reproductive biology in captivity. The objective of the present study was to determine altrenogest (Regumate ®) post-treatment indicators of vaginal cytology, estradiol levels and restarting of reproductive activity of *T truncatus* females in captivity in marine environment. Twelve females received altrenogest at a daily dose of 0.07mg kg^{-1} for a year. A total 420 slides of vaginal cytology of each female were performed to determine the percentage of cornified cells. Also, 60 blood samples of each animal were analyzed to determine estradiol levels. Regarding the vaginal cytology; percentage of cornified cells increased between 60 and 70% from day 4 to day 9 after removing the altrenogest treatment and between 70 and 80% from day 12 to day 19. Estradiol levels were in the range of 16 to 114pg ml^{-1} during the entire monitoring period. A positive correlation ($r = 0.7062$; $P<0.05$) was found between these indicators. Therefore, we conclude that treatment with altrenogest and monitoring the estrous cycle with simple techniques such as vaginal cytology might be used for designing protocols for assisted reproduction for groups of *T truncatus* in captivity.

Keywords: Altrenogest, Bottle-Nosed, estrous, reproduction.

ACTIVIDAD REPRODUCTIVA DESPUÉS DEL ANESTRO INDUCIDO CON ALTRENOGEST EN HEMBRAS DE *Tursiops truncatus* EN CAUTIVERIO EN AMBIENTE MARINO

RESUMEN

La necesidad de reproducir delfín nariz de botella (*Tursiops truncatus*) en cautiverio se ha incrementado debido a las restricciones internacionales para su comercialización y por el riesgo y dificultad logística para el traslado de ejemplares. Por lo anterior, se hace

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importante conocer su biología reproductiva en cautiverio. El objetivo de este trabajo, fue conocer los indicadores post tratamiento con altrenogest (Regumate®), de citología vaginal, niveles de estradiol y reinicio de la actividad reproductiva en cautiverio de hembras de *T truncatus* en ambiente marino. Por un año, 12 hembras recibieron diariamente una dosis de 0,07mg kg^{-1} de altrenogest. Se realizaron un total 420 citologías vaginales, una diaria de cada hembra, para determinar el porcentaje de células cornificadas. También se obtuvieron de la red vascular, 60 muestras sanguíneas, en las que se determinó los niveles de estradiol. En cuanto a la citología vaginal, al cuarto día de retirar la administración de altrenogest, el porcentaje total de células cornificadas incrementó del 60% a 70% hasta el día nueve y continuó ascendiendo al 80% entre los días 12 al 19. Los niveles de estradiol, presentaron un rango de 16 a 114pg ml^{-1}. Se encontró una correlación ($r = 0,7062$ $P<0.05$) positiva entre estos indicadores. Se concluye que es posible la manipulación con altrenogest y el monitoreo del ciclo estral de las hembras mediante técnicas simples como la citología vaginal para el diseño de protocolos de reproducción asistida específicos para grupos en cautiverio de *T truncatus*.

Palabras clave: Altrenogest, delfín, estro, reproducción.

INTRODUCTION

Since 1938, with the first reports of bottlenose dolphins (*Tursiops truncatus*) kept in captivity, there has been interest to understand and optimize their reproduction in captivity (Biancani *et al.* 2009; Katzumata 2010; O´Brien *et al.* 2009; O´Brien y Robeck 2010; Teraswa *et al.* 2008). It has been observed that reproductive activity in captivity can be influenced by local microclimate which alters female reproductive physiology mainly in specimens coming from a different geographical origin (Montie *et al.* 2008; Robeck *et al.* 2009; Urian *et al.* 1996). Therefore, it is clear there is a need for monitoring reproductive activity of each population in captivity for adequate reproductive handling and for the successful implementation of assisted reproduction techniques (O´Brien y Robeck 2010; Robeck *et al.* 1994). The application of artificial insemination in *T truncatus* has required studies to know and manipulate their estrous cycle in order to reversibly suppress ovarian activity to synchronize or inhibit estrous in female specimens. This has been frequently achieved by oral administering Altrenogest (Regumate ®) (Biancani *et al.* 2009; Katsumata 2010; O´Brien y Robeck 2010; Robeck *et al.* 2001). Altrenogest used for inhibiting reproductive activity does not seem to have adverse effects on reproduction and/or behavior of the females of *T truncatus*.

During the estrous cycle, which is associated with the modification of different hormone levels, mainly estradiol and Lh, there are cellular changes on the vaginal epithelium. One of these changes is the desquamation and keratinization into the lumen of the more external layers of the vaginal epithelium due to a increase on the estradiol concentrations at the end of estrous. This is the reason why the vaginal smear of the stages immediately after the estradiol peaks is characterized by cornified, flat and irregular cells without nucleus (Hafez y Hafez 2000). In studies performed on different populations of *T truncatus* kept in captivity, their estrous cycles have been characterized by measuring hormone levels (Katsumata 2010; Robeck *et al.* 2005). This kind

of studies, besides being invasive (blood samples are required) are very expensive because laboratory procedures such as radio-immunoassay (RIA) to determine hormone levels are not always available near the locations where the specimens are kept. Therefore, the objective of the present study was to determine the altrenogest post-treatment changes of vaginal cytology, estradiol levels and restart of reproductive activity of *T truncatus* females kept in captivity in marine environment.

MATERIALS AND METHODS

Twelve *T truncatus* females with an age of 12.4 ± 2 years (range 10-15 years old) with at least one birth, were monitored from September to October The average of temperature was 27.4 °C and 26.6 °C, respectively. Specimens were located in the Mexican Caribbean at the geographical coordinates 20°26´39.5´´ N. 86°59´39.7´´W, kept in group in captive facilities of wire mesh sea pens. Females had four sessions of 40 minutes daily of interactive swim with people. Specimens were fed with frozen fish (*Clupea arengus, Mallotus villosus, Menidis spp*) having a daily consumption of approximately 60 to 80kcal kg^{-1}.

Hormonal treatment

Females received altrenogest at a daily dose of 0.07mg kg^{-1} body weight with their food, taking in account the doses recommended in a previous study to inhibit reproductive activity and behavior (Robeck *et al*. 2005; Robeck *et al*. 2012). The treatment was administered for at least 12 months before the study and it was stopped simultaneously on the same day for all females in the study.

Animal welfare

Specimens were trained before the study to avoid stress and risks associated with the physical contention, mobilization and handling that could expose the physical integrity of the specimens or the personal. Samples for vaginal cytology, estradiol serological determinations and ultrasonography were obtained using the operating conditioning protocol always based in positive reinforcements (Lenzi 2000). All procedures described herein were approved by the Dolphin Discovery Institutional Animal Care and Use Committee, and were performed in accordance with the Animal Welfare Act for the care of Marine Mammals.

Vaginal cytology/ estradiol levels

A total of 420 samples for vaginal cytology were collected, 60 before stopping the altrenogest treatment and 360 in the30 days after removing the treatment. One daily sample was obtained for each specimen with an interval of 24 hours. Also, after removing the treatment, 60 blood samples were obtained for determining estradiol levels; five samples were obtained from each specimen with intervals of five days between samples for causing no harm to the animals by the punctures needed for getting the blood.

A) Samples for vaginal cytology were taken from each female by introducing a sterile swab at least 20cm into the vagina after drying the external genital area. The swab was washed in 5 ml Hartman solution for 20seg; 25µl of the solution obtained after the washing were prepared by the Papanicolaou staining technique to determine the proportion of cornified cells. Cornifed cell were those squamous, enucleated and with an irregular shape.

B) Blood samples were drawn from the periarterial venous rete (Bossart *et al.* 2000), with a 19-gauge, 1.9 cm butterfly catheter (Becton Dickinson). Serum was collected in 10 ml separator vacutainer tubes (Becton Dickinson) and centrifuged for 15 min at 1200 rpm (350 ×g). In order to use an available methodology for getting fast results, estradiol determination was performed by the quimioluminiscence, method (Hitachi 911) in a commercial laboratory.

A statistical analysis was performed to estimate the simple correlation between the proportion of cornified cells and the estradiol concentrations in the serum of the females after removing the altrenogest treatment. Data for estradiol levels and percentages of cornified cells were analyzed by descriptive statistics using the range as measure of dispersion and by a simple linear regression analysis using the software JMP5.

RESULTS

The vaginal cytology from samples taken before stopping the treatment with altrenogest showed between 30 to 45% of cornified cells. After removing the treatment, three peaks of increase on the percentage of cornified cells were observed. The first peak was observed at the eighth day with an increase up to 80%, followed by a decrease reaching 41%, four day later. The second peak showed a constant increase higher than the first one, reaching 82% between days 14 and 22 and a reduction of 49 % was observed at day 24. The third peak reached an 89% of cornified cells between days 25 to 28; immediately after which a clear and abrupt decrease was observed reaching the lowest proportion of cornified cells (17%). Regression analysis showed a linear increase ($p<0.0001$) in the number of cornified cells as estradiol concentration increased (Figure 1). This result is supported by the positive correlation ($n=60$; $r=0.7062$; $P<0.05$) between the cornified cells and estradiol concentrations (Figure 2). Variations on the percentages of cornified cells were preceded by increases on serum estradiol concentrations (from 24 to 48 hours before). Estradiol concentrations ranged between 18 to 112pg ml^{-1}. Three peaks were identified: the first with a maximum value of 80pg ml^{-1}between the fifth and eighth day, the second of a longer duration (from day 14 to 22) presented a maximum value of 112pg ml^{-1} and the third peak observed between days 26 to 28 had a maximum value of 89pg ml^{-1}.

DISCUSSION

These results were different to those obtained in other studies where reproductive activity was restarted until the 21st day after stopping the altrenogest treatment (Robeck *et al.* 1994; Robeck *et al.* 2001). Reproductive activity in the other nine specimens restarted at different times, but it was always associated to an increase in estradiol levels followed by an increase of the percentage of cornified cells in the vaginal cytology. This relation presented a pattern where the increase of cornified cells was observed from 24 to 48 hours after the increase in estradiol concentrations.

Hormonal levels measured in the present work by the quimioluminiscence methodology can be useful as a tool to determine and characterize the reproductive physiological state of *Tursiops truncatus* females. Moreover, the obtained values were similar to those reported in other studies with females of the same species kept in captivity (Estradiol, 23-90 pg ml^{-1}) determined by RIA (Sawyer-Stefan *et al.* 1983; Schneyer *et al.* 1985).

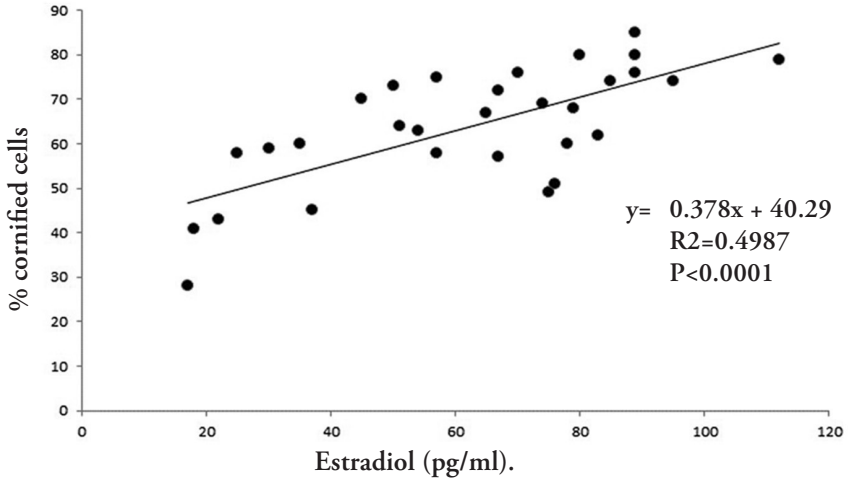

FIGURE 1. Lineal regression between estradiol serum concentrations and percentage of cornified cells in Tursiops truncatus vaginal cytology. [Regresión lineal simple entre las concentraciones séricas de estradiol y el porcentaje de células conrnificadas en citologías vaginales de. Tursiops truncates]

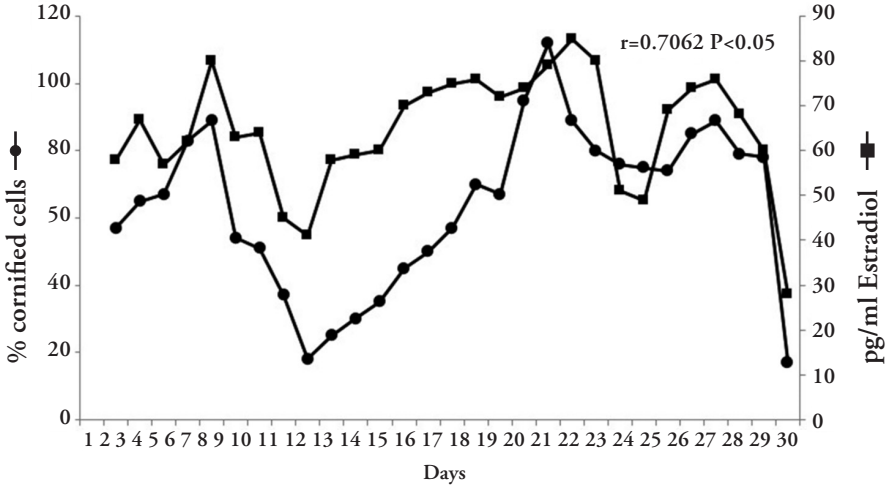

FIGURE 2. Increases and decreases the percentage of cornified cells and estradiol levels during the sampling period showing positive correlation. [Incrementos y disminuciones de los porcentajes de células cornificadas y niveles de estradiol durante el periodo de muestreo que muestran correlación positiva]

Furthermore, the use of both assays to determine estradiol levels was also corroborated by the clear peaks on the proportion of cornified cells preceded by the increase in estrogens concentrations. In another study, it was also demonstrated that the increase in estrogen levels induced changes in the vaginal mucusa producing a progressive queratinization of the layers of the vaginal epithelium finding from 80 to 100% of queratinized cells in a moment very close to ovulation (Muraco *et al.* 2004).

The present results show that vaginal cytology is a useful tool of easy access which provides important and reliable information to identify the different stages of the estrous cycle and the reproductive activity of *T truncatus.* This represents a contribution for the development of programs for assisted reproduction which intend to understand the reproductive physiology of this species in order to preserve it in captivity (Schneyer *et al.* 1985).

The implementation of rapid diagnostic methods to achieve programs for assisted reproduction in captivity are useful for preventing diseases such as the papillomavirus associated with a great quantity of tumorations present in specimens in captivity and even in those wild and free conditions. The results of the present study demonstrate that with the use of altrenogest and monitoring the estrous cycle of the females using simple techniques that vaginal cytology, it is possible contribute to the development of more efficient protocols for assisted reproduction of *T truncatus,* as it has been already proposed in others studies (Robeck 2004; Robeck *et al.* 2009; O´Brien y Robeck 2010). An important application for these protocols is to be able to program offspring births to avoid the hurricane season that represent a risk for specimens in all sites located in the Caribbean Sea.

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