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# *A protocol for gynecological and obstetric examination of owl monkeys using ultrasound*

## *Protocolo para exame ginecológico e obstétrico por ultrassom em macacos-da-noite*

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**ABSTRACT:** The objective of this study was to establish a physical restraint protocol for gynecological and obstetric examination using ultrasound in *Aotus azarai infulatus*. The study was conducted with 10 previously established adult couples, evaluated during two different time periods (P1 and P2). In P1, the conditioning was carried out by daily repetition of the gynecological exam associated with the offering of fruits before, during and after the exam. In P2, gynecological examinations were performed on non-pregnant females (n = 6), and obstetric examinations on those who became pregnant (n = 3). The exams were performed using the Medical SonoAce 9900®ultrasound equipment equipped with a multifrequential linear array probe (5-12 MHz). The initial and final heart rate (HR1 and HR2) for each female were calculated based on the interval between systolic peaks. No significant differences were observed in the heart rates in terms of time of examination, suggesting that the time did not interfere in the variables analyzed during the exams. The gynecological and obstetric exams presented a HR2 lower than HR1 during the two periods evaluated ($p<0.01$). The stress caused by initial restraint increased sympathetic activity and raised HR1. Offering fruits and habituating the animals favored the parasympathetic modulation, thereby explaining the decrease in HR2. This was observed in both gynecological and obstetric exams.

**RESUMO:** O objetivo deste estudo foi estabelecer um protocolo de contenção física para a realização do exame ginecológico e obstétrico e ultrassom em macacos *Aotus azarai infulatus*. O estudo foi realizado com 10 casais de adultos previamente estabelecidos, avaliados em dois períodos de tempo diferentes (P1 e P2). No P1, o condicionamento foi realizado pela repetição diária do exame ginecológico, associado à oferta de frutas antes, durante e após o exame. Em P2, foram realizados exames ginecológicos em fêmeas não prenhes (n = 6) e obstétricos para aquelas que ficaram gestantes (n = 3). Os exames foram realizados com o equipamento de ultrassom Medical SonoAce 9900®, equipado com uma sonda linear multifrequencial (5-12 MHz). A frequência cardíaca inicial e final (HR1 e HR2) de cada fêmea foi calculada com base no intervalo entre os picos sistólicos. Não foram observadas diferenças significativas na frequência cardíaca em relação ao tempo de exame, sugerindo que o tempo não interferiu nas variáveis analisadas durante os exames. Os exames ginecológicos e obstétricos apresentaram HR2 inferior à HR1, durante os dois períodos avaliados ($p <0,01$). O estresse causado pela contenção inicial aumentou a atividade simpática e elevou a HR1. A oferta das frutas e a habituação dos animais favoreceram a modulação parassimpática, justificando a queda na HR2. Isso foi observado em exames ginecológicos e obstétricos.

## 1 Introduction

Members of the genus *Aotus*, commonly referred to as owl monkeys, have been used historically in biomedical research to address a wide range of scientific questions. These moderately small, nocturnal Neotropical primates possess several unique features that make them ideal for use as an animal model of human disease. These features include susceptibility to infection with *Plasmodium* spp., large eyes with easily visualized retinas, adaptability to captivity, and relative ease of handling (BAER, 1994).

One of the major drawbacks to the use of nonhuman primates (NHP) is that they can be very difficult and even dangerous to handle; restraint is therefore necessary and desirable to protect both the investigator and the animal. Primates in the laboratory many experience a plethora of potential stressors including physical and chemical restraint, venipuncture, injection, and participation in other husbandry routines such as catching, changing cages, and weighing. Training them to cooperate voluntarily, using positive reinforcement training (PRT) techniques, is one means of significantly reducing the adverse impact of such procedures and husbandry routines on them and, this is therefore an improvement (PRESCOTT; BUCHANAN-SMITH, 2003).

The use of anesthetic drugs and the capture method used to perform ultrasonographic monitoring of gestation in owl monkeys acted as chemical and environmental factors, resulting in abortion (MONTEIRO et al., 2006). But, some small primates (Callitrichids) can be trained to be handled and hence to be scanned without sedation (OERKE et al., 1996). Voluntary cooperation reduces the need for physical restraint and/or anesthesia and, therefore, also the risks associated with those events (LAULE et al., 2003). Therefore, according to Monteiro et al. (2006), the establishment of methods for conditioning the female owl monkey to the ultrasound exam may provide a solution to this problem.

Therefore, the objective of this study was to establish a physical restraint protocol for gynecological and obstetric examination using ultrasound in owl monkeys.

## 2 Materials and Methods

The animals were born in captivity and belonged to the breeding colony of the National Primate Center (CENP), located in the municipality of Ananindeua, in the Brazilian State of Pará, Brazil (latitude 1°38'26" and longitude 48°38'22"). The experimental project was approved by the Ethics in Research with Animals Committee of the Evandro Chagas Institute (protocol CEPAN / IEC – n° 047/2005). Each animal was identified with a three-letter code tattooed on the inner part of the right thigh and a microchip placed in the interscapular area.

The study was conducted with 10 previously established adult owl monkey (*Aotus azarai infulatus*) couples, evaluated during two different time periods (P1 and P2). The animals were four to ten years old, housed indoors, in Sector I (for monogamic species), in brick-built enclosures, covered with tiles and wire netting. The cages measured 3.85 m in length x 1.20 m width x 2.40 m height. Externally, the enclosures had a 35 cm shelter box of 35 cm x 30 cm x 30 cm, with a guillotine type door and a water bottle for drinking. Internally, there were two wooden platforms for walking and access to the food bowls. The animals were fed according to CENP husbandry practices: a variety of fruits, vegetables, milk, eggs, dry dog food, vitamin and mineral supplements and water *ad libitum*.

In the first period (P1), the females (n = 10) were isolated from the males for four months, and examined daily to condition them to the ultrasound examination. The conditioning was achieved by daily repetition of the gynecological exam associated with the offering of fruits before, during and after the exam. After this period, the males were taken to the female enclosures where they remained for 30 days, although without ultrasound evaluation.

The second period (P2), starting 30 days after P1, involved mating and the females (n = 9) were examined once a week, for seven consecutive months. Gynecological examinations were performed on non-pregnant females (n = 6) and obstetric examinations on those who became pregnant (n = 3).

During P1, 522 gynecological ultrasound exams were performed, and, during P2, 195 gynecological and 50 obstetric examinations (Figure 1). The criteria used for these exams were similar to those described in the literature (MONTEIRO et al., 2006, 2009).

The females were captured by a keeper who was instructed to enter the enclosure, place a fruit inside the shelter box and encourage the female

to enter the box. The box was then closed by a guillotine door. The female was transferred to a wooden transportation box (35 cm x 30 cm x 40 cm) and taken to the ultrasound room.

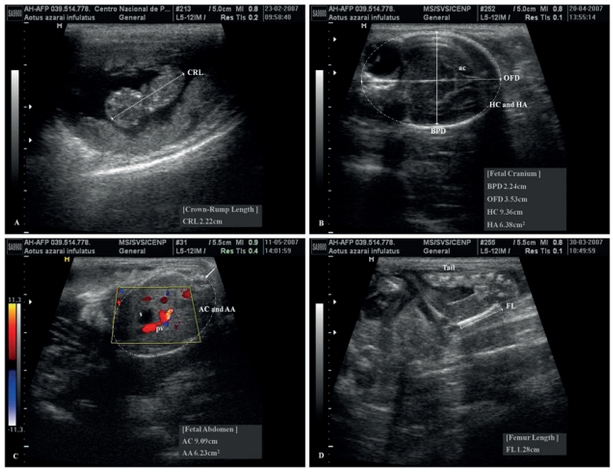


**Figure 1.** (A) The crown-rump length (CRL) measurement in the eighth week of gestation. (B) The plane of the thalamic exam in the 16th week of gestation, showing the ambient cistern (ac) in the posterior section of the head. The biparietal diameter (BPD) was obtained by positioning the probe from the external surface of the proximal cranial table to the distal internal surface. The occipitofrontal diameter (OFD) was obtained by measuring the external margins of the fetal cranium, perpendicular to the BPD. The head circumference and head area (HC and HA) were obtained during the same examination by a tracing around the external border of the hyperechogenic outline in the fetal cranium border. (C) A color Doppler image during the 19th week of gestation was used to facilitate identification of the umbilical portion of the portal vein (pv) in the liver and the fetal stomach. The abdominal circumference and the abdominal area (AC and AA) were measured using fetal abdomen transverse scan, perpendicular to the vertebral axis (arrow). (D) The femur length (FL) in the 13th week of gestation was measured from the proximal and distal extremities, measuring only the femoral diaphysis.

Before starting the ultrasound examination, the females were weighed using a Filizola® MF-30 scale and manually restrained, and the pelvic-abdominal area was shaved to remove hair to facilitate ultrasound evaluation. Various kinds of fruit were offered to the animals before, during and after the procedure. The main fruits offered were avocado, pineapple, banana, guava, papaya, watermelon and melon. The fruits were sliced longitudinally into pieces of approximately 10cm in length, to avoid accidents (bites) during feeding.

The exams were performed using the Medical SonoAce 9900® ultrasound equipment (Medison) equipped with a multifrequential linear array probe (5-12 MHz). The images obtained were analyzed on a 14"monitor, printed on thermal paper with a Sony® VP 895 MD video graphic printer ( Sony Corporation, Tokyo, Japan) and stored on a CD-Rom.

Initial and final heart rates (HR1 and HR2) for each female were calculated on the basis of the interval between systolic peaks, obtained in Spectral Doppler Mode in the iliac arteries (Figure 2). The average heart rate (HRA) for each exam was taken to be the arithmetic mean of HR1 and HR2.

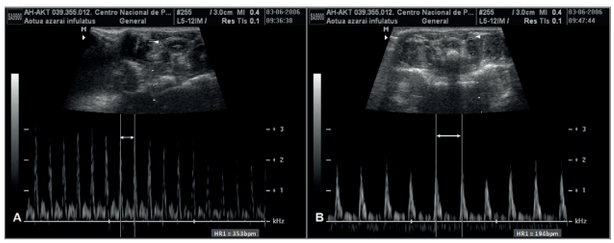


**Figure 2.** Heart rate evaluation in Spectral Doppler Mode in the iliac arteries (arrow), calculated based on the distance between systolic peaks (double arrows). (A) Initial Heart Rate (HR1 = 353 bpm), (B) Final Heart Rate (HR2 = 194 bpm), emphasizing the reduction of 159 bpm compared to HR1.

The initial time (IT) of the exam was registered at the moment that the female was removed from the transportation box and the final time (FT) when she was returned to the box. The time spent on the exam (TE) was calculated as the difference between FT and IT. Using these data, a comparison of the average rates of HR1, HR2 and HRA, correlated with TE, was established for the two periods (P1 and P2).

The examinations in P1 and P2 were conducted at three different times of day: T1 (8:00 to 11:59 a.m.), T2 (12:00 to 4:59 p.m.) and T3 (5:00 to 7:59 p.m.), with a view to correlating the time of day of the examination with an increase or decrease in heart rate.

The mean HR1 and HR2, in the same period, were compared using the t-test. The means of the HR1, HR2, HRA and TE variables were compared at three different times of day (T1, T2 and T3), and between P1 (gynecological exams) and P2 (gynecological and obstetric exams), using One Way ANOVA. The individual means were then compared using the Tukey test. The Pearson correlation test was used to compare TE and HR2 in the two periods. The results were considered statistically significant at a 5% level of significance.

## 3 Results

The females were easily conditioned to enter the shelter and transportation boxes. However, behavior indicative of stress was displayed during the physical restraint. The animals vocalized, urinated, defecated and tried to escape during restraint. In many cases, urination and defecation were observed during the transportation to the location where the exam was going to be performed or when the transportation box was opened.

During the experiment it was observed that the number of repeated examinations and the offering of fruit during restraint were important factors in reducing stress indicators. This can be explained by the fact that, even without quantification of these indicators, a reduction could be seen. This suggests that the animals became habituated to the ultrasound exam when it was repeatedly performed.

It was also possible to identify a preference for pineapple and watermelon, compared to the other fruits offered. The act of slicing the fruits into pieces of approximately 10cm in length reduced the risk of biting, as it enabled a greater distance to be maintained between the animal's oral cavity and the hand of the person performing the exam.

No significant differences were observed in the heart rates regarding the time of day of the examination (T1, T2 and T3), suggesting that this did not interfere in the variables analyzed during the gynecological and obstetric exams.

The data presented in Table 1 show that the gynecological and obstetric exams presented HR2 lower than HR1 during the two periods evaluated ($p<0.01$). In some examinations, differences of up to 159 bpm between HR1 and HR2 (Figure 2) were identified.

**Table 1.** Heart rate and time spent on the examination (mean ± standard deviation) during the periods P1 and P2.

| Variables | P1 | P2 | |
|---|---|---|---|
| | Gynecological exam | Gynecological exam | Obstetric exam |
| HR1 (bpm) | 323.3aA ± 49.44 | 313.8aA ± 43.65 | 326.8aA ± 31.15 |
| HR2 (bpm) | 292.4bAB ± 53.32 | 282.0bBC ± 52.95 | 301.7bA ± 32.40 |
| HRA (bpm) | 307.9AB ± 45.98 | 297.8BC ± 40.84 | 314.2A ± 26.22 |
| TE (min.) | 18.51A ± 7.13 | 13.78B ± 3.30 | 18.31A ± 7.22 |

HR1 – initial heart rate; HR2 – final heart rate; HRA – average heart rate; TE - time spent on the exam. Lower-case letters mean that the comparison was carried out by row, between HR1 and HR2 (by t test). Upper-case letters mean that the comparison was carried out by column (using One Way ANOVA). Different letters in the same line or columns indicate $p<0.05$.

The comparisons established between the gynecological exams performed during the two periods demonstrated that the HR1, HR2, and HRA obtained in P2 were lower than those obtained in P1, but this was not statistically significant ($p>0.05$).

The obstetric exams (Figure 1) performed in P2 demonstrated higher heart rates than those observed in the gynecological exams in P1 and P2. However, significant differences ($p<0.05$) were only observed in the comparisons established between HR2 and HRA of the gynecological exams performed in P2.

The time spent (TE) to perform gynecological examinations in P1 was significantly greater than in P2 ($p<0.001$), but this did not occur in the case of the obstetric exams (Table 1). The correlation tests (r) applied for TE and HR2 indicated low level correlation. The gynecological exam in P1 presented $r = - 0.1249$ ($p = 0.1937$). When the same exam was performed in P2, $r = - 0.1740$ ($p = 0.0130$) was observed and, during the obstetric exam in P2, $r = - 0.01352$ ($p = 0.9265$). This indicates that there was a tendency for HR2 to decrease while TE increased.

## 4 Discussion

The method of offering fruits served as a positive reinforcement leading the animals to associate the exam with something pleasurable and stimulating (eating). This is in accordance with Schapiro et al. (2005) who mention the offering of food as a positive reinforcement in bringing about a desired behavior. The same authors report that reinforcement using food or liquids is called primary, since this has immediate biological consequences. This can be compared to the decrease in the number of stress indicators observed in our experiment. The beneficial effects of training animals to collaborate in scientific research are also described by other authors (PRESCOTT; BUCHANAN-SMITH, 2003; BENTSON et al., 2003; REINHARDT, 2003; SCHAPIRO et al., 2005; CAPITANIO et al., 2006).

Animal behavior is controlled by the endocrine and nervous systems. The complexity of this characteristic is related to the complexity of the nervous system. The primates, therefore, have a greater capacity to learn new experiences and adjust their behavior accordingly. The analysis of the results showed that the behavior indicative of stress is in accordance with the findings of Monteiro et al. (2006), who reported urination and defecation during physical restraint in owl monkeys.

The variation in the heart rate was the only stress indicator quantified and showed similar results when compared to other indicators, since it decreased significantly in the two time periods analyzed. These findings are fairly similar in animals and human beings, and can be explained by the "flight-or-fight" mechanism triggered by the autonomic nervous system and by the action of the hypothalamic-adenohypophysis-adrenocortical pathway (HALL et al., 2004; GUYTON; HALL, 2006).

Some studies carried out in humans and laboratory animals indicate that the autonomic neurones and the increase in plasma concentrations of the Adrenocorticotropic hormone (ACTH) act on the motility of the intestinal colon promoting defecation or diarrhea (OKANO et al., 2005). Variation in the heart rate is a parameter for evaluating neurocardiac functioning, since the autonomic modulation, through the sympathetic and parasympathetic pathways, influence the oscillation of this variable in a direct and differentiable manner (MALIK, 1996).

An increase in heart rate is, according to the literature, the cardiovascular response most commonly related to stress (HERD, 1991). The results presented in this study therefore indicate that the stress caused by the initial restraint promoted an increase in the sympathetic activity and raised the initial heart rate (HR1). At the end of the exam, the positive reinforcement related to the offering fruits and habituation of the animals favored the parasympathetic modulation and this would explain the decrease in the final heart rate (HR2). This was observed in the case of both gynecological and obstetric exams.

In humans, there is evidence that vagus nerve stimulation has a protective effect on ventricular electric vulnerability, and a low parasympathetic activity could be correlated with the development of lethal arrhythmia (REIS et al., 1998). Radiotelemetry studies carried out in *Callithrix jacchus, Macaca mulatta and M. fascicularis* suggest that blood pressure and heart rate are significantly higher when animals are subjected to stress, even if previously trained for such procedures (SCHNELL; WOOD, 1993; SCHNELL; GERBER, 1997).

During the obstetric exams, heart rates higher than those recorded during the gynecological exams in P1 and P2 were observed. It can, therefore, be inferred that, during gestation, the females were more prone to stress, even though they were previously conditioned. These findings may be related to those of studies conducted by Barron et al. (1986) that suggested that regular gestations alter cardiovascular responses by way of stimulation of the sympathetic nervous system.

Some authors have observed greater activity of the hypothalamic – hypophisary- adrenal pathway in pregnant females compared to non-pregnant ones (MATTHEWS; RODIN, 1992) and greater susceptibility to stress in early gestation than in later stages (GLYNN et al., 2001).

Another hypothesis that may explain the increase in heart rate during the obstetric exams is the increase in blood flow, basal metabolism and maternal breathing during gestation. There is an increase of 30 to 40% in blood flow and heart deficiency in women, at 27 weeks of gestation. Shortly thereafter, for reasons that remain unknown, the heart deficiency drops to slightly above normal during the last eight weeks of pregnancy, despite the high uterine blood flow. Due to the increased basal metabolism in pregnant women, the total amount of oxygen used by the mother, shortly before birth, is about 20% above normal. Simultaneously, the volume of the uterus increases, compressing the diaphragm and causing an increase in the mother's minute ventilation (GUYTON; HALL, 2006). These physiological alterations that occur during the gestation may therefore be responsible for the compensatory increase in heart rate.

The negative correlations observed suggest that an increase in TE causes a decrease in HR2, favoring parasympathetic system activity. The TE was appropriate for the ultrasound examination, according to the principle that duration of restraint must be the minimum necessary for performing the desired procedure (FOWLER, 1986). The significant differences relating to the TE when compared to the gynecological exam in P1 and P2 were attributed to poorer conditioning of the females in P1, as they were less habituated to the ultrasound. According to the literature, habituation is a kind of learning that animals develop in response to a frequently repeated stimulus (PRESCOTT; BUCHANAN-SMITH, 2003; SCHAPIRO et al., 2005; CAPITANIO et al., 2006). The reduction in the risk of biting through use of the technique of slicing fruit is in accordance with the findings reported by Fowler (1986), who remarks that safety (of the animal and the researcher/assistant) and the type of restraint must be carefully evaluated and planned before it is employed.

## 5 Conclusion

Training NHP to cooperate rather than resist during handling procedures provides a simple improvement on traditional, involuntary restraint techniques. The results presented in this study have shown that restraining female monkeys and conditioning methods were important in enabling gynecological and obstetric examinations to be performed, for study of the reproductive organs and the chronological development of owl monkey fetuses.

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