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Ultrasound evaluation of pregnancy in owl monkey (*Aotus azarai infulatus*)

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Abstract

Pregnancy in owl monkeys was monitored using ultrasonography. The obstetric exams were performed using a 5-12 MHz linear probe. The measurements included the mean gestational sac diameter (MGSD), crown-rump length (CRL), biparietal diameter (BPD), occipitofrontal diameter (OFD), head circumference (HC), head area (HA), abdominal circumference (AC), abdominal area (AA) and femur length (FL). The Pearson's correlation coefficient showed highly significant positive correlations between most of the fetal growth variables ($r > 0.90$, $P < 0.01$). The AC x FL and AA x FL correlations did have correlation coefficients <0.90, although this value was still highly significant ($P < 0.01$). These results suggest that all of the fetal growth variables are strongly associated with each other.

Keywords: *Aotus sp*, conceptus development, pregnancy, ultrasonography.

Introduction

The genus *Aotus* (owl monkey) is one of 110 species and subspecies found in the Amazon (Rylands *et al*., 2000), 53 of which are endemic (Machado *et al*., 2005). However, the diversity of this fauna coexists with the advance of deforestation and the destruction of the natural resources resulting from human activity, which has led to habitat degradation and the consequent conservation difficulties faced by primate species. The timber activity in rainforests is certainly one of the major factors that contribute to this threat, in addition to hunting and the commercial exploitation of some species (Coutinho *et al*., 2011).

Despite these issues, little is known about the reproductive physiology of Brazilian Amazon primates. The few basic studies have been behaviorally based. Traditionally, owl monkeys have been considered to be delicate and difficult to breed in captivity. The majority of laboratories have used these animals for research on malaria or cancer, but little attempt has been made to study their reproductive biology (Dixson, 1994). In the last decade, there have been some descriptions of owl monkey reproduction in the literature (Monteiro *et al*., 2006, 2009; Valle *et al*., 2006; Schuler *et al*., 2007, 2010; Coutinho *et al*., 2011). However, an improved knowledge of primate reproductive physiology is necessary.

Ultrasonography is a routine imaging modality that is used for colony management and experimental protocols at many nonhuman primate (NHP) facilities. The reproductive applications of ultrasound provide an efficient method for detecting pregnancy, monitoring the fetus during gestation, and the routine assessment of breeding females (Tarantal, 2005).

Several authors have reported that NHPs follow the general plan of mammalian embryo development and that many of the events that are related to the beginning of gestation are structurally and chronologically similar to those in human gestation (Nyland *et al*., 1984; Conrad *et al*., 1989; Corradini *et al*., 1998; Bourry *et al*., 2005).

Although it often requires anesthesia in NHPs, ultrasonography is both safe and noninvasive. It can be used to estimate the gestational age and the date of parturition and to detect placental and fetal abnormalities (Lee *et al*., 1991; Schuler *et al*., 2007). However, owl monkeys are susceptible to stress when ultrasound monitoring is needed. The use of anesthetic drugs and the capture method may act as chemical and environmental factors that jeopardize gestation monitoring in this species (Monteiro *et al*., 2006). Therefore, the objective of this study was to monitor the gestation of owl monkeys using ultrasound to stimulate further research and answer questions related to the gestational physiology of this species.

Materials and Methods

Nine previously established owl monkey (*Aotus azarai infulatus*) couples were utilized in this study; the females were 4-10 years old, and the males were 5-13 years old. The animals belonged to the breeding colony of the National Primate Center (CENP), which is located in Ananindeua county, state of Pará, Brazil (latitude 1°38'26" and longitude 48°38'22"). All the study procedures were approved by the Evandro Chagas Institute ethical committee (protocol CEPAN/IEC - n° 047/2005).

The females were captured using the method of Monteiro *et al*. (2009). The keeper was instructed to enter the enclosure and place a fruit inside a shelter box

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to entice the female to enter the box. The box was then closed by a guillotine door. The female was transferred to a wooden transport box (35 cm x 30 cm x 40 cm) and moved to the ultrasonography room. Before starting the ultrasonographic examination, the animals were weighed using a Filizola® MF-30 scale (Indústrias Filiziola S/A. Rua Joaquim Carlos, 1236, São Paulo, SP, Brazil) and manually restrained, and all of the pelvic-abdominal area was clipped to remove hair and facilitate the ultrasound assessment.

The females were kept isolated from the males for four months and examined daily to condition them to the ultrasound examination. Fruits were offered to the animals before, during and after the procedure. The fruits offered were avocado, pineapple, banana, guava, papaya, watermelon and melon. The conditioning was accomplished by the daily repetition of the gynecological exam associated with offering the fruit (i.e., by positive reinforcement).

After this period, the males were taken to the female enclosures, where they remained for seven months. We did not perform any exams on the females during the first month of mating, but the copulatory behavior was monitored every day. From the second month, each female was examined once a week for seven months, and a total of 245 examinations were performed: 195 in nonpregnant females (n = 6), and 50 in healthy pregnant females (n = 3).

The ultrasonographic exams were performed using the SonoAce 9900® ultrasound equipment (Medison Co., Ltd, Medison Venture Tower, 997-4. Daechi-dong, Kangnam-ku, Seoul, 135-280, Korea), equipped with a multifrequential linear array probe (5-12 MHz). The images obtained were analyzed on a 14" monitor and recorded on thermic paper linked to a Sony® VP 895 MD video graphic printer (Sony Corporation, Tokyo, Japan) and on CD-ROM media.

The exams attempted to identify the early and developmental ultrasonographic signs of pregnancy. The early signs of pregnancy were defined as endometrial thickness (ET) and uterine volume (UV) increases. The ET evaluation attempted to detect an echogenicity increase in the uterine fundus region at the thickest point between the two basal layers on the anterior and posterior uterine walls. The ET evaluation was performed three times to calculate the mean ET value (Fig. 1A). To define the uterine volume, we compared the median values for pregnant and nonpregnant females, taking the craniocaudal (CCD), dorsoventral (DVD) and transverse (TD) diameters into consideration (Fig. 1A). A single examiner assessed each uterine variable three times to calculate the mean UV.

The date of conception was calculated from the mating observations and with the mean duration of gestation described in the literature. According to some authors, owl monkey gestation is approximately 132-135 days (19 weeks; Dixson, 1994; Málaga *et al*., 1997). The first week of gestation was defined by the visualization of early signs (ET and uterine volume). The mean gestational sac diameter (MGSD; Fig. 1B) and further embryonic/fetal growth parameters were considered developmental signs of pregnancy. Each subsequent week was evaluated until term, which was the last week of gestation. The first trimester of gestation consisted of the period between the first and the sixth weeks. The second trimester began in the seventh week and extended through the twelfth week. The third trimester was defined as the period between the thirteenth week and the nineteenth week, when parturition occurred.

The embryonic development was evaluated by the MGSD, which was calculated by the arithmetic mean of the length and height (by sagittal scan) and width (by transversal scan) variables (Fig. 1B) and by the crown-rump length (CRL) measurement of the embryo (Fig. 1C). Both of these variables were evaluated according to the method of Sauerbrei *et al*. (2000). The following factors were the main fetal development parameters:

1. The biparietal diameter (BPD) and the occipitofrontal diameter (OFD) were measured in the thalamic plane, which was determined by the ambient cistern appearance in the rear of the head. The BPD was measured by placing the calipers from the external surface of the proximal cranial table to the distal internal surface. The OFD was obtained by measuring the distance from one external fetal cranium margin to the other margin perpendicular to the BPD (Fig. 1D).
2. The head circumference and head area (HC and HA, respectively) were measured by the delineation of the fetal cranium's hyperechoic outline in the same scan that was used for the BPD and OFD (Fig. 1D).
3. The abdominal circumference and abdominal area (AC and AA, respectively) were measured by placing the probe around the external portion of the fetal abdomen's hyperechogenic border. To do this, a transverse scan, perpendicular to the vertebral axis, was obtained from the fetal abdomen. This scan visualized the stomach and the umbilical portion of the portal vein in the liver. The color Doppler mode was used to easily identify the umbilical portion of the portal vein (Fig. 1E).
4. The femoral length (FL) was measured from its distal and proximal diaphysis extremities (Fig. 1F).

The criteria used to obtain the images and measure the gestational parameters were similar to those described in the literature (Herring *et al*., 1991; Sauerbrei *et al*., 2000; Monteiro *et al*., 2006). Each variable was measured weekly by a single observer six times, and these values were used to calculate the mean and standard deviation. We calculated the linear (Pearson) correlation matrix for the vector of fetal growth variables (BPD, OFD, HC, HA, AC, AA, FL). We considered correlations to be statistically significant at a 1% significance level (Steel *et al*., 1997).

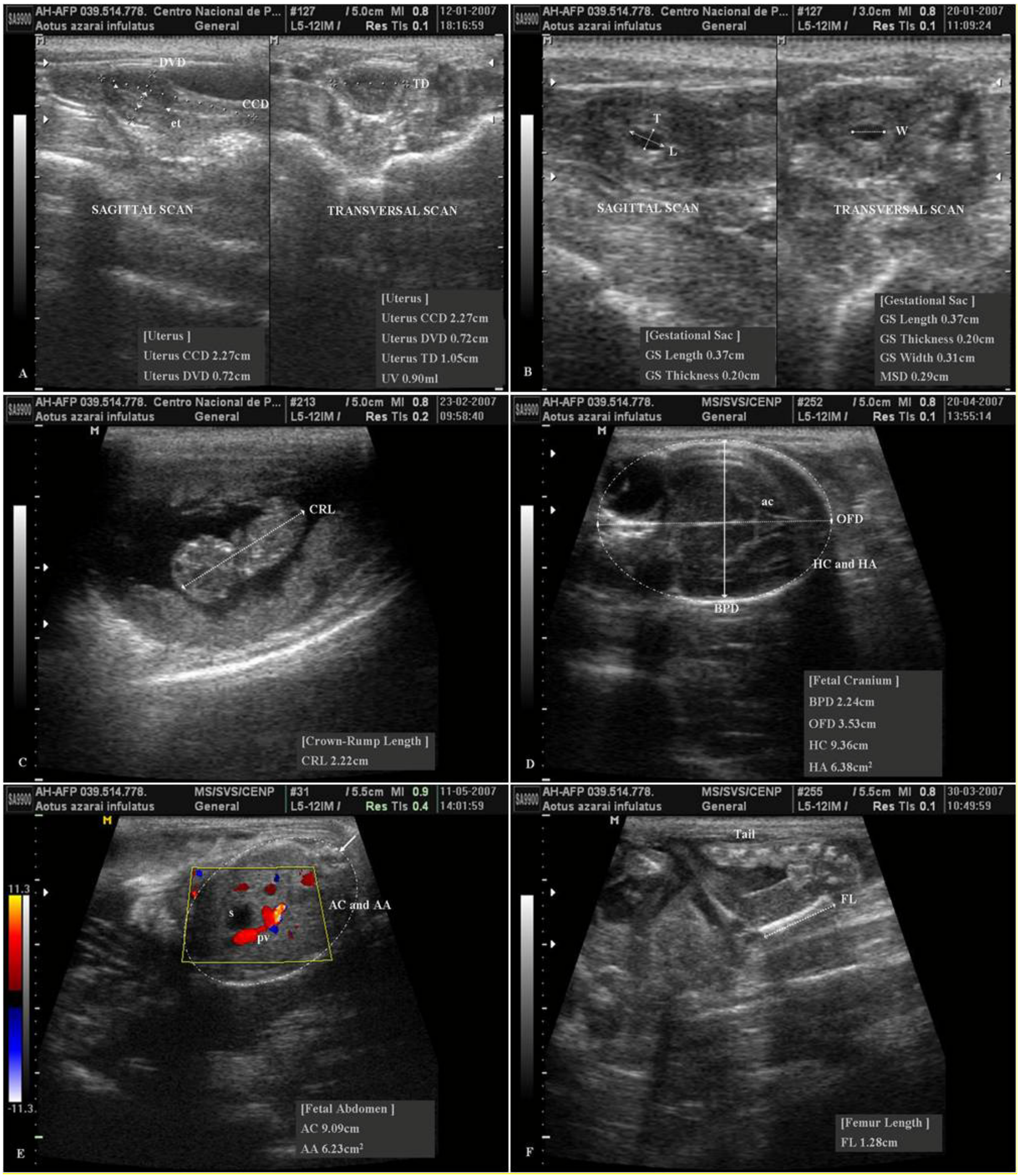

Figure 1. (A) The second week of gestation, showing the increase in the uterine variables (CCD, DVD, TD and UV) and the endometrial thickness (ET), which are considered early gestational signs. The ET was measured at the thickest point between the two basal layers on the anterior and posterior uterine walls. (B) The fundus implantation of the GS in the third week of gestation of the same female, showing the mean gestational sac diameter (MGSD), length (L), and height (H) by sagittal scan and the width (W) by transverse scan. (C) The crown-rump length (CRL) measurement in the eighth week of gestation. (D) 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 in the same examination by a tracing around the external border of the hyperechogenic outline in the fetal cranium border. (E) A color-Doppler image during the 19th week of gestation that was used to facilitate identifying the umbilical portion of the portal vein (pv) in the liver and the fetal stomach (s). The abdominal circumference and the abdominal area (AC and AA) were measured in the fetal abdomen transverse scan, perpendicular to the vertebral axis (arrow). (F) The femur length (FL) in the 13th week of gestation was measured from the proximal and distal extremities, measuring only the femoral diaphysis.

Results

Three positive gestations (33%) were diagnosed during the experimental period, of which two were carried to term and one was spontaneously interrupted at the beginning of the third trimester of gestation (between the 12^{th} and 13^{th} weeks). The second and third trimesters were characterized by progressive increases in the fetal growth parameter values until parturition.

We diagnosed the gestations from the second month of mating. The early signs of pregnancy (ET and increase of uterine volume) and development (MGSD and CRL) were evaluated in the first trimester. The ET was 0.32 ± 0.04 cm (mean ± standard deviation) in the two first weeks of gestation (Fig. 1A) and was 0.08 ± 0.05 cm when the females were not pregnant. The ultrasound monitoring indicated that the pregnant females showed a significant increase in UV (0.91 ± 0.29 ml) compared with the nonpregnant females (0.51 ± 0.15 ml) during the first two weeks of gestation. These findings were important for determining the early signs of pregnancy in the owl monkeys. However, a conclusive diagnosis in the three gestations was only possible through visualizing the developmental signs that began in the third week with the appearance of the anechoic gestational sac in the uterine fundus region (Fig. 1B).

The second trimester was defined as the interval between the 7^{th} and 12^{th} weeks. During this period, the MGSD (until the eighth week) and CRL (until the tenth week) were measured. We obtained BPD, OFD, HC and HA measurements from the eighth week onward. The AC and AA were measured from the ninth week onward, and the FL was measurable only from the tenth week onward.

We began to visualize the embryo proper only in the fourth week. The MGSD and CRL measurements during the first trimester of gestation were obtained from the third to eighth and the fifth to tenth weeks, respectively. Their growth patterns are shown in Table 1.

Table 1. The main embryonic/fetal growth parameters (mean ± standard deviation) were evaluated weekly during the first, second and third trimesters of pregnancy in *Aotus azarai infulatus*.

Period of gestation (week)		Biometric Parameter								
		MGSD (cm)	CRL (cm)	BPD (cm)	OFD (cm)	HC (cm)	HA (cm^2)	AC (cm)	AA (cm^2)	FL (cm)
First trimester (n = 3)	1	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-
	3	0.31 ± 0.02	-	-	-	-	-	-	-	-
	4	0.62 ± 0.02	-	-	-	-	-	-	-	-
	5	1.03 ± 0.11	0.48 ± 0.02	-	-	-	-	-	-	-
	6	1.37 ± 0.05	0.83 ± 0.09	-	-	-	-	-	-	-
Second trimester (n = 3)	7	1.93 ± 0.04	1.40 ± 0.05	-	-	-	-	-	-	-
	8	2.84 ± 0.21	2.27 ± 0.11	0.87 ± 0.04	1.09 ± 0.03	3.14 ± 0.08	0.77 ± 0.04	-	-	-
	9	-	3.83 ± 0.18	1.09 ± 0.05	1.42 ± 0.05	4.03 ± 0.11	1.25 ± 0.07	2.72 ± 0.13	0.57 ± 0.04	-
	10	-	5.05 ± 0.07	1.30 ± 0.05	1.67 ± 0.05	4.76 ± 0.10	1.78 ± 0.14	3.79 ± 0.01	1.15 ± 0.04	0.55 ± 0.02
	11	-	-	1.52 ± 0.05	2.03 ± 0.16	5.72 ± 0.33	2.52 ± 0.26	4.55 ± 0.17	1.63 ± 0.11	0.82 ± 0.01
	12	-	-	1.67 ± 0.04	2.32 ± 0.20	6.44 ± 0.45	3.17 ± 0.36	5.46 ± 0.27	2.32 ± 0.22	1.04 ± 0.06
Third trimester (n = 2)	13	-	-	1.87 ± 0.05	2.83 ± 0.03	7.57 ± 0.12	4.23 ± 0.20	6.33 ± 0.07	3.03 ± 0.04	1.31 ± 0.04
	14	-	-	1.98 ± 0.01	2.96 ± 0.07	7.91 ± 0.26	4.70 ± 0.12	7.63 ± 0.21	4.20 ± 0.26	1.52 ± 0.02
	15	-	-	2.07 ± 0.00	3.24 ± 0.05	8.63 ± 0.27	5.35 ± 0.50	8.44 ± 0.43	5.32 ± 0.72	1.68 ± 0.03
	16	-	-	2.25 ± 0.01	3.46 ± 0.09	9.03 ± 0.47	6.05 ± 0.47	8.25 ± 0.07	4.90 ± 1.85	1.85 ± 0.03
	17	-	-	2.34 ± 0.03	3.77 ± 0.26	9.89 ± 0.59	7.03 ± 0.58	8.58 ± 0.44	5.67 ± 0.40	1.98 ± 0.01
	18	-	-	2.45 ± 0.08	3.98 ± 0.41	10.70 ± 0.58	8.09 ± 0.70	9.00 ± 1.17	6.16 ± 1.40	2.15 ± 0.03
	19	-	-	2.46 ± 0.00	3.93 ± 0.00	10.54 ± 0.00	8.11 ± 0.00	9.09 ± 0.00	6.23 ± 0.00	2.31 ± 0.00

MGSD, mean gestational sac diameter; CRL, crown-rump length; BPD, biparietal diameter; OFD, occipito-frontal diameter; HC, head circumference; HA, head area; AC, abdominal circumference; AA, abdominal area; FL, femur length.

We performed 29 BPD, OFD, HC, and HA measurements during the three pregnancies (between the 8th and 19th weeks of gestation). These parameters increased with the gestational age (Table 1). The AC and AA variables were measured 26 times during the two term pregnancies (between the ninth and 19th weeks), and the FL was measured 23 times in them between the 10th and 19th weeks. Therefore, a progressive increase in the size of the parameters with gestational age that appeared to slow towards the term was observed.

The correlation coefficients (r) between the fetal growth variables showed highly significant positive correlations (r > 0.90, P < 0.01) between most of the variables (Table 2). The AC x FL and AA x FL correlations did have correlation coefficients <0.90, although this value was still highly significant (P < 0.01). These results suggest that all of the fetal growth variables are strongly associated with each other (i.e., as one variable increases, so do the rest).

Table 2. The correlation coefficients (r) between the fetal growth variables (BPD x OFD x HC x HA x AC x AA x FL) in *Aotus azarai infulatus*.

Variables	BPD	OFD	HC	HA	AC	AA	FL
BPD	1.00	0.990**	0.992**	0.975**	0.975**	0.966**	0.995**
OFD		1.00	0.998**	0.990**	0.969**	0.968**	0.983**
HC			1.00	0.991**	0.965**	0.963**	0.985**
HA				1.00	0.948**	0.958**	0.980**
AC					1.00	0.990**	0.664**
AA						1.00	0.814**
FL							1.00

BPD, biparietal diameter; OFD, occipito-frontal diameter; HC, head circumference; HA, head area; AC, abdominal circumference; AA, abdominal area; FL, femur length. **P < 0.01.

Discussion

The results indicate that ultrasound is a reliable method for observation of obstetric measurements during prenatal development in *Aotus azarai infulatus*. Similar data were described in *Callithrix jacchus* by Jaquish *et al*. (1995). Moreover, the early diagnosis of gestation in NHP is important for clinical and investigative reasons. For researchers who use these animals as models of human reproduction, the ability to diagnose gestation at around 14 days after conception would create new opportunities for physiological and pathological studies (Conrad *et al*., 1989). Determining gestational age is an important tool in primate breeding colonies. Measurements of embryonic/fetal growth variables can be used to determine gestational age in New and Old World monkeys (Shimizu, 1988; Tarantal, 1990, 2005; Herring *et al*., 1991; Jaquish *et al*., 1995; Corradini *et al*., 1998; Bourry *et al*., 2005), including the owl monkey (Monteiro *et al*., 2006; Schuler *et al*., 2010). However, this study is the first to describe many of these variables in owl monkeys; Schuler *et al*. (2010) described only the correlation between BPD measurements and gestational age in *Aotus nancymaae*.

Consistent with the literature, the endometrial thickness and uterine volume increase were considered early signs of pregnancy but were not sufficient to establish a precise gestational diagnosis because, in humans and NHPs, these variables may undergo pathological and physiological alterations that affect the endometrial echogenicity and uterine volume when the female is not necessarily pregnant (Conrad *et al*., 1989; Sauerbrei *et al*., 2000; Monteiro *et al*., 2006). Therefore, visualizing the gestational sac was necessary to confirm the diagnosis and to establish gestation development in our study. However, it is important to emphasize that there was a progressive increase in the uterine volume as gestation development became evident.

In chimpanzees and baboons, the gestational sac is visible by ultrasonography three weeks after conception, and the embryo is visible after four weeks (Herring *et al*., 1991; Hobson *et al*., 1991). Studies of the gestation of *Saguinus fuscicollis* using ultrasound have verified that, after conception, the endometrial surfaces become separated and form a lumen with a hypoechogenic aspect (a double endometrial echo) that is detectable between 16 and 18 days of gestation (17.2 ± 1.0 days). The presence of the gestational sac has been verified between the fourth and sixth weeks after conception (41.8 ± 7.1 days, an interval of 32-52 days; Kuederling and Heistermann, 1997). This structure has also been verified between the second and third weeks of gestational age in capuchin monkeys (15-22 days; Corradini *et al*., 1998). Studies of callitrichid species have established that the double endometrial echo ensures the safety and precocity of the ultrasound gestation diagnosis. This echo occurred within 15 days of gestation in *C. jacchus*, 17 days in *S. fuscicollis* and *S. oedipus,* and in 12 days in *Callimico goeldii* (Oerke *et al*., 2002).

A previous study in owl monkeys has indicated that the gestational sac and the embryo proper appear between 28 and 38 days after mating (Monteiro *et al*., 2006). In our study, we found that the double endometrial echo was visualized and measured in gestations at the beginning of gestational sac formation, which occurred from the third week of gestation. The embryo proper was detected from the fourth week. These findings are similar to those that have been described for chimpanzees, baboons, and owl monkeys (Herring *et al*., 1991; Hobson *et al*., 1991; Monteiro *et al*., 2006). The gestational sac measurement can be used for evaluating gestational age until the eighth week of pregnancy in owl monkeys. Subsequently, the gestational sac size exceeds the area covered by the probe scan field. Similar data have also been reported in macaque and capuchin monkeys (Tarantal and Hendrickx, 1988; Corradini *et al*., 1998).

In common marmoset, triplets were significantly ($P < 0.05$) smaller than twins for both BPD and CRL. No significant relationship was found between litter size and within litter variation in CRL or BPD (Jaquish *et al*., 1995). A later study with fifty *C. jacchus* pregnancies resulted in the hypothesis that marmosets may be able to adjust litter size late in pregnancy in response to proximate environmental factors (Jaquish *et al*., 1996). In the present study there were no twin or triplets pregnancies, but these data are important, because a case of successful survival of twins in *Aotus* was described in the literature (Málaga *et al*., 1991).

According to Nyland *et al*. (1984), the *Macaca mulatta* fetus presents a CRL of 0.60 ± 0.60 cm in the third week and 6.60 ± 0.60 cm in tenth week. In this species, the CRL growth process may be slightly different than in humans. These results were similar to our study because we measured this variable in the fifth (0.48 ± 0.02 cm) and tenth (5.05 ± 0.07 cm) weeks of gestation. However, there is a tendency for accelerated growth in owl monkeys because the gestational period is shorter (19 weeks).

In rhesus monkeys, the reported BPD, HC and HA measurements have begun in the seventh week (Nyland *et al*., 1984). According to Tarantal (2005), the predicted BPD values for rhesus and long-tailed macaques are observed in the late first to third trimesters (50-165 days gestation). In capuchin monkeys, the BPD provided an accurate estimate of gestational age from 45 days of gestation until parturition. In *Aotus nancymaae*, measuring the BPD is possible from 40 days gestation until the end of pregnancy, which occurs at approximately 19 weeks (Schuler *et al*., 2010). We obtained measurements related to the fetal skull (BPD, OFD, HC and HA) from the eighth week (56 days) until the 19th week of gestation, when pregnancies went to term. Therefore, the BPD measurements obtained in our study were similar to those described for *A. nancymaae* (Schuler *et al*., 2010).

According to Nyland *et al*. (1984), it is possible to measure the AC and AA in rhesus monkeys from the seventh week of pregnancy until the delivery date. These data are similar to the measurements described in this study. In our owl monkeys, however, these variables were only measured from the ninth week of gestation.

In the owl monkeys, the fetal FL can easily be measured from the tenth week of pregnancy onward by the increased echogenicity of the bone, which provides a useful gestational variable. In capuchin (Corradini *et al*., 1998) and rhesus monkeys (Nyland *et al*., 1984), the FL has been measured on days 70 and 73, respectively, which matches the growth linearity of the tenth week of pregnancy in these species. By contrast, Tarantal and Hendrickx (1988) have shown that the FL can be measured from 47 days of gestation in *M. fascicularis* and *M. mulatta*.

In summary, this study presented the first complete ultrasonographic evaluations of early pregnancy and fetal development in *Aotus azarai infulatus*. We described nine variables for predicting the gestational age in weeks. These data may be useful in studies of reproductive physiology in species of the genus *Aotus* and as tools for the managers of owl monkey breeding colonies. However, new studies on gestational monitoring in this species should be encouraged and aim to provide regression equations for estimating the gestational age in days.

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