INTERPOPULATIONAL VARIATION IN REPRODUCTIVE CYCLES AND ACTIVITY OF THE WATER SNAKE *LIOPHIS MILIARIS* (COLUBRIDAE) IN BRAZIL

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This study reports on aspects of reproduction in the water snake *Liophis miliaris* from four regions in Brazil: (1) northern coastal Atlantic forest, (2) southern coastal Atlantic forest, (3) northern inland Atlantic forest; and (4) southern inland Atlantic forest. In the northern coastal Atlantic forest, where there is little climate variation, the reproductive cycle of this species is continuous, with vitellogenesis and oviposition occurring throughout the year. Newly hatched snakes are found mainly in January. In other regions the cycle is seasonal and related to warmer and rainy periods, with vitellogenesis and oviposition occurring mainly from September to February. Hatchlings are more abundant from February to April, at the end of the rainy season. In the northern Atlantic forest newly hatched snakes have smaller body sizes than in the other regions. Sperm production seems to occur throughout the year in all regions, and where reproduction is seasonal, mating seems to be disassociated from vitellogenesis, suggesting that sperm may be stored by females over the winter. Females with oviductal eggs did not feed, whereas those ones with secondary vitellogenic follicles fed more frequently than non-reproductive females. In all regions, the activity pattern of adult *Liophis miliaris* seems to be related to reproductive cycles and climate variation.

Key words: geographic variation, reproduction, seasonal activity, Serpentes

INTRODUCTION

Snakes can reproduce seasonally even in tropical areas (Shine, 2003). In most Neotropical species vitellogenesis starts in the early rainy season (September-October), egg-laying occurs throughout the latter part of this season (January-February), and hatching occurs mostly at the end of the rainy season and onset of the dry season (March-May) (*cf.* Marques, 1996*a*; Marques & Puorto, 1998; Fowler *et. al.*, 1998; Hartmann *et al.*, 2002; Marques, 2002; Pinto & Fernandes, 2004). In viviparous snakes, female reproductive cycles are usually seasonal but there is not a single pattern in the timing of vitellogenesis and parturition (Sazima, 1992; Almeida-Santos & Salomão, 1997; Bizerra *et al.*, 2005; Aguiar, 2002; Almeida-Santos & Orsi, 2002; Oliveira *et al.*, 2003). On the other hand, some oviparous species (e.g. *Erythrolamprus aesculapii, Xenodon neuwiedii, Oxyrhopus guibei*) display continuous cycles (Marques, 1996b; Jordão, 1996, Pizzatto & Marques, 2002), but reproductive peaks can occur in the rainy season (*cf.* Pizzatto & Marques, 2002). Spermatogenic cycles are less well known but apparently both seasonal and continuous patterns can occur (Janeiro-Cinquini *et al.*, 1993; Pizzatto & Marques, 2002; Shine, 2003).

As in other reptiles, reproductive cycles in snakes can be related to climatic variation (*cf.* Seigel & Ford, 1987).

In temperate areas, reproduction is non-continuous and limited by seasonal temperature cycles, whereas in the tropics it can be related to both temperature and rainfall (Seigel & Ford, 1987). In tropical habitats where variation in temperature throughout the year is minimal but rainfall is usually seasonal, snakes tend to reproduce in the rainy season (e.g. Vitt & Vangilder, 1983; James & Shine, 1985; Shine *et al.*, 1998). Therefore, reproductive patterns are high variable in the tropics mostly due to climatic complexity (Greene, 1997). As a result, widespread tropical species are good models for studying the effect of climatic parameters on reproduction.

Liophis miliaris belongs to the colubrid tribe Xenodontini and occurs mainly in forested areas from southern Guiana to Argentina (Dixon, 1989). It is associated with aquatic habitats and feeds mainly on anurans and fishes (Vitt & Vangilder, 1983; Michaud & Dixon, 1989; Sazima & Haddad, 1992; Marques & Souza, 1993). Previous studies of *Liophis* have suggested the probability of continuous reproductive cycles in species from the Brazilian caatinga (northern Brazil – Vitt & Vangilder, 1983) and also in the Amazon region (Martins & Oliveira, 1998). However, *Liophis miliaris* from southeastern Brazil is suspected to have a seasonal cycle (Albolea, 1998; Marques 1998; Marques & Sazima, 2004). The aim of this study was to obtain data for the purpose of testing two main hypotheses: (1) that *L. miliaris* may reproduce continuously and reproductive patterns can differ among populations in different geographic areas, and (2) that

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reproductive patterns in *L. miliaris* are related to climatic parameters such as temperature and rainfall.

MATERIAL AND METHODS

POPULATIONS STUDIED

We studied four distinct populations of the water snake *Liophis miliaris* (Fig. 1) from the following areas:

1. Northern Coastal Atlantic Forest (NCAF): this population occurs in south of Bahia state (13°48'N, 18°04'S, 30°08'E and 40°43'W, Fig. 1). Currently this area is managed for the cultivation of cacao, which is grown inside the rainforest. The climate is aseasonal with high temperatures and rainfall throughout the year (Fig. 2).

2. Southern Coastal Atlantic Forest (SCAF): located in São Paulo-Paraná sates, (23°26'N, 25°52'S, 45°04'E and 48°50'W), on the east of Serra do Mar. This region is mostly covered by rainforest. Spring and summer (September to March) are usually rainy with high temperatures, and autumn-winter (April to August) is warm and dry (Fig. 2).

3. Northern Inland Atlantic Forest (NIAF): located in São Paulo state, (20°12'N, 24°41'S, 45°06'E and 51°06'W), in the west of Serra do Mar. This area is mainly covered by semi-deciduous seasonal forest. Climatic variation is similar to SCAF but monthly fluctuations are more pronounced (Fig. 2).
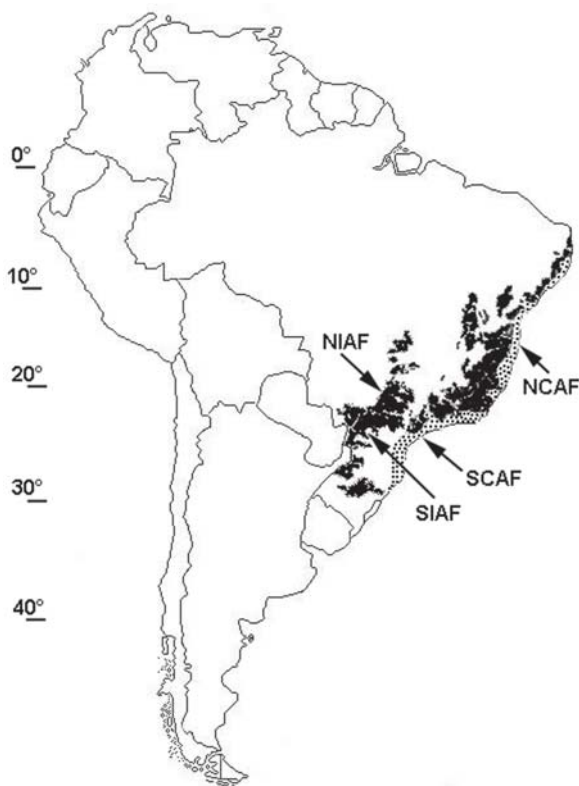

FIG. 1. Distribution of the *Liophis miliaris* populations studied in Brazil. NCAF = Northern Coastal Atlantic Forest, SCAF = Southern Coastal Atlantic Forest, NIAF = Northern Inland Atlantic Forest, SIAF = Southern Inland Atlantic Forest. Dark areas = Inland Atlantic Forest domain, dotted areas = Coastal Atlantic Forest domain.

4. Southern Inland Atlantic Forest (SIAF): located in Paraná state, (22°51'N, 27°17'S to 45°18'E and 54°35'W). This area is covered by the semi-deciduous seasonal forests and by the Araucaria forest. Spring-summer (September to March) is usually hot but temperatures are lower than in the other areas. Autumn-winter (April to August) tends to be cold with abundant rainfall throughout the year and no clear dry season (Fig. 2).

MORPHOLOGICAL MEASUREMENTS

Liophis miliaris exhibits marked variation in colour pattern and according to the most recent review (Dixon, 1989) it is represented by seven subspecies. Following this classification, the population from NCAF is representative of *L. m. merremii*, whereas populations from the remaining areas studied (SCAF, NIAF, and SIAF) are *L. m. orinus*. Moreover, individuals from SCAF differ in colour pattern from NIAF and SIAF and possibly belong to distinct taxa (see Gans, 1964; Marques *et al.*, 2004). Thus, these populations may represent three distinct subspecies (and not to two, as previously proposed by Dixon, 1983; 1989). Alternatively, these individuals may belong to two or three different species of the genus *Liophis*, a possibility which could be confirmed only after a taxonomic revision of the group. In light of these unresolved taxonomic issues, we use the most recent classification as proposed by Dixon (1983, 1989), and consider the four populations of *L. miliaris* defined in this study which at least are representative of a single monophyletic group.

We examined a total of 289 preserved specimens of *Liophis miliaris* (127 adult females, 140 young and 22 adult males) from NCAF; 249 (84 adult females, 106 young and 59 adult males) from SCAF; 167 (49 adult females, 80 young and 38 adult males) from NIAF and 201 (84 adult females, 64 young and 53 adult males) from SIAF. These specimens are held in the collections of Museu de Zoologia da Universidade de Santa Cruz (MZUESC), Comissão Executiva do Plano da Lavoura Cacaueira (CEPLAC), Museu de História Natural da Universidade Estadual de Campinas (ZUEC), Instituto Butantan (IB) and Museu de História Natural do Capão da Imbuia (MHNCI). We measured snakes SVL to the nearest 1 mm, and after dissection recorded the following additional data: (1) reproductive condition - mature or immature (females were considered mature if they had follicles in secondary vitellogenesis (>10 mm in diameter), oviductal eggs or folded oviducts (Shine, 1977*a*); males were judged to be mature if the testes were enlarged and turgid, or if the deferent ducts were opaque and convoluted, indicating the presence of sperm (see Shine, 1977*b*); (2) diameter of the largest ovarian follicles or eggs in females (measured with a vernier calliper to the nearest 0.1 mm); (3) oviductal condition: folded or not (much folded oviducts indicate recent egg-laying); (4) length, width and thickness of the testes (measured with a vernier calliper to the nearest 0.1 mm), testicular volume (TV), as estimated using
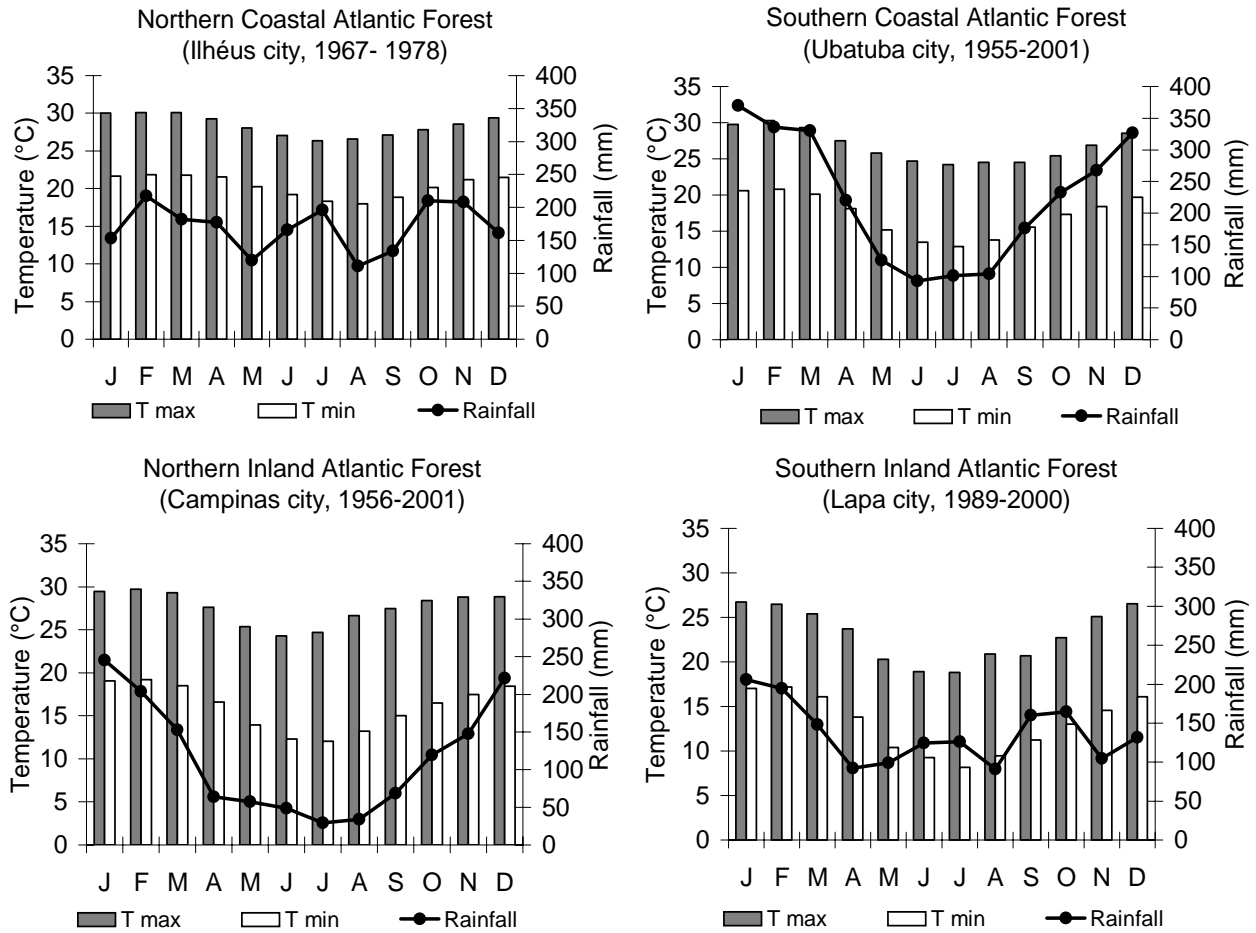
FIG. 2. Climatic patterns in the northeastern and southeastern Brazilian areas where the studied populations of *Liophis miliaris* were collected.

the ellipsoid volume formula TV = 4/3*abc* where *a* = half of length, *b* = half of width and *c* = half of thickness (see Pleguezuelos & Feriche, 1999); (5) deferent duct diameter close to the cloacae (measured with a vernier calliper to the nearest 0.1 mm), and (6) presence of prey in the stomach

Testis volume and deferent duct diameter indicate spermatogenic activity and sperm release and storage (Volsøe, 1944; Fox, 1952; Shine, 1977*b*; Marques *et al.*, 2004). Snake testicular volume and deferent duct diameter were related to SVL and these allometric relations vary throughout the reproductive cycles (i.e. throughout the year – Shine, *et al.* 1998). Thus, we calculated the residuals of the regressions of these measures by SVL in the four seasons: January-March, April-June, July-September and October-December. Residuals were then compared in a Kruskal-Wallis test to identify differences on these variables within the seasons (Zar, 1999).

OVIPOSITION AND ACTIVITY PERIODS

We gathered data on the period of egg-laying from seven females which were collected while they were gravid (detected by palpation) and maintained in captivity (without males) until oviposition. Captive individuals were maintained at room temperature

(around 19 - 31 °C), with water provided *ad libitum* and food (fishes and frogs) offered every ten days.

The number of neonates from SCAF and NIAF received monthly at Instituto Butantan were used as estimative of recruitment in these regions (*cf.* Marques *et al.,* 2001, but see also Shine, 1980*a*). In the remaining areas the number used was the number of neonates collected during each month.

Climatic diagrams were supplemented with data collected from localities in the areas studied. These data were obtained from CEPLAC, Instituto Agronômico de Campinas and Instituto Agronômico do Paraná. We inferred snake activity by comparing the number of individuals collected in each of the four seasons (see Shine, 1980*a* and Marques *et al.,* 2001 for use and comments on this method) using a Chi-square test (Zar, 1999). Relationships between number of snakes collected per month (dependent variable), minimum temperature and mean rainfall were investigated by multiple correlations (Zar, 1999).

RESULTS

FEMALE REPRODUCTIVE CYCLE

Females with vitellogenic follicles, oviductal eggs or much folded oviducts were found throughout the year in

TABLE 1. Percentage of fed females, their reproductive status and evidence of multiple clutches in *Liophis miliaris* from Brazil. Pizzatto & Marques, Reproduction and activity in *Liophis miliaris*, NCAF = Northern Coastal Atlantic Forest, SCAF =Southern Coastal Atlantic Forest, NIAF =Northern Inland Atlantic Forest, SIAF = Southern Inland Atlantic Forest.

|	NCAF	SCAF	NIAF	SIAF
Fed females	17% (n=22)	9.5% (n=8)	12.2% (n=6)	10% (n=8)
Fed females in secondary vitellogenesis	36.4% (n=8)	50% (n=4)	83.3% (n=5)	87.5% (n=7)
Fed females pregnant	0	0	0	12.5% (n=1)
Multiple clutches		one captive female laid eggs in January 2000, December 2000 and February 2001; one preserved female with oviductal eggs and vitellogenic follicles (18.5 mm).	two captive females laid eggs in intervals between two months and 11 months; one preserved female with oviductal eggs and vitellogenic follicles (16.3 mm).	four females with oviductal eggs and vitellogenic follicles (10.7; 10.9; 11.1 and 12 mm respectively).
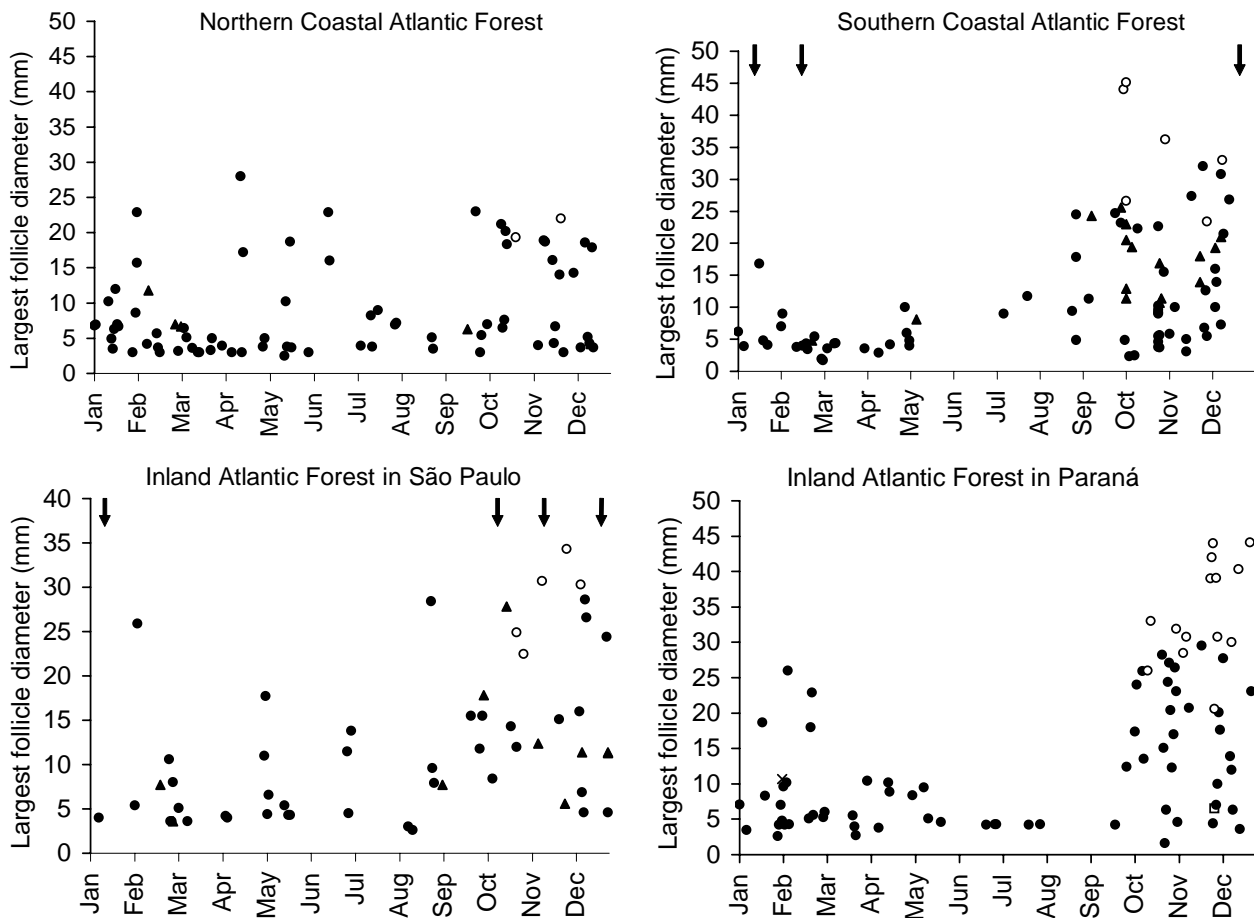
FIG. 3. Seasonal variation in the diameter (in mm) of the largest follicle (full circles) in *Liophis miliaris*. Empty circles = oviductal eggs, triangles = follicle and folded oviducts, cross = follicle and *corpora lutea,* rows = oviposition.

NCAF but only from September to January in SCAF and NIAF, and from October to February in SIAF (Fig. 3). Evidence of multiple clutches was found in all populations except in NIAF (Table 1). In general, pregnant females do not feed (Table 1) but females in secondary vitellogenesis had prey items in stomach more frequently than non-reproductive females (Table 1).

In NCAF, newly hatched snakes (SVL< 160 mm – see Pizzatto & Marques, 2005 for hatchling sizes) were recorded throughout the year but mainly in January (Fig. 4). In the other three areas, newly-hatched snakes (SVL < 230 mm) were more abundant between February and April (Fig. 4).

TESTICULAR CYCLE

None of studied populations showed significant variation, among the four seasons, in the residual volume of testis (Kruskal-Wallis test - SCAF: HL. Pizzatto and O. A. V. Marques H=0.079, P= 0.994, n=52; NIAF: 0.432, P=0.933, n=37; SIAF: H=0.010, P=0.999, n=48) or deferent duct diameter (Kruskal-Wallis test - SCAF: H=0.343, P=0.952, n=55; NIAF: 0.113, P=0.990, n=37; SIAF: H=0.414, P=0.916, n=48), indicating a continuous spermatogenic cycle. The sample of

males (n=22) from NCAF was of insufficient size to permit analysis.

ACTIVITY

Adult females were most commonly collected from January to March in NCAF (χ^2=11.1, df = 3, P=0.011, Fig. 4), from October to February in SCAF (χ^2=33.4, df =3, P<0.0001; Fig. 4), from October to December in NIAF (χ^2=15.1, df=3, P<0.0017; Fig. 4) and also in SIAF (χ^2=45.0, df=3, P<0.0001; Fig. 4). Number of females collected per month is positively correlated to minimum temperature in NCAF (R^2=0.60, P=0.016, $\beta_{(min\ temp)}$=1.79, t =3.50, P=0.007; $\beta_{(mean\ rainfall)}$= -0.01, t= 0.52, P=0.615) and also in SIAF (R^2=0.41, P=0.089, $\beta_{(min\ temp)}$=1.24, t=2.32, P=0.045; $\beta_{(mean\ rainfall)}$= -0.02, t= 0.47, P=0.652), but not in SCAF (R^2=0.33, P=0.920) or NIAF (R^2=0.15, P=0.527).

Adult males were most commonly collected in SCAF from October to February (χ^2=19.8, df=3, P=0.0002; Fig. 4) and from January to March in SIAF (χ^2= 10.5, df =3, P=0.0149; Fig. 4). The number of collected males did not vary in NIAF (χ^2=1.6, df=3, P=0.676; Fig. 4). Number of males collected per month was not correlated with minimum temperature or mean rainfall in any of the regions (SCAF: R^2=0.57, P=0.193; NIAF:
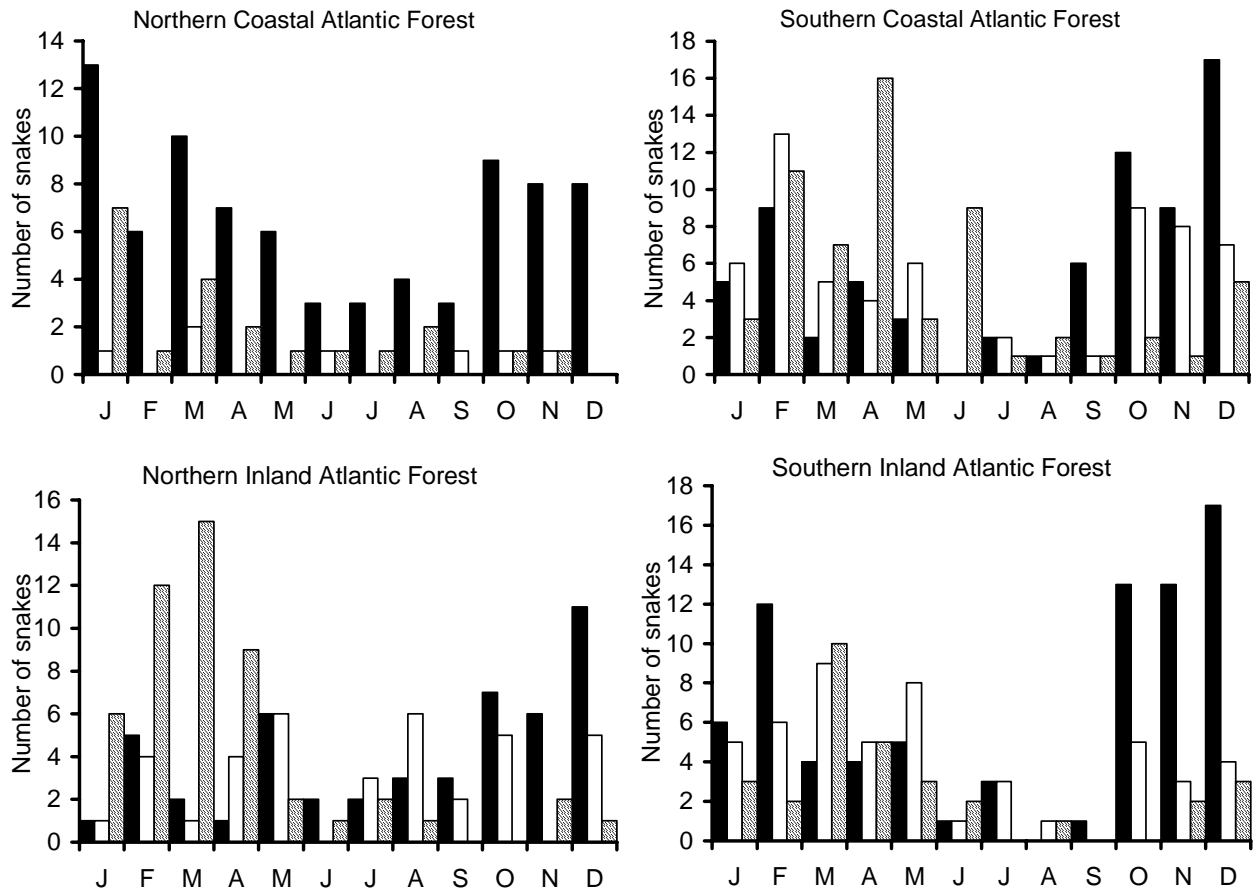

FIG. 4. Seasonal collection of *Liophis miliaris* from the studied populations in Brazil. White bars = adult males, black bars = adult females, striped bars = hatchlings.

$R^2=0.12$, $P=0.555$; SIAF: $R^2=0.28$, $P=0.221$). The sample of males from NCAF was too small to analyse.

DISCUSSION

FEMALE REPRODUCTIVE CYCLES

In the NCAF, the climate is homogeneous throughout the year, which may allow constant food availability (Begon *et al.*, 1990). Such a condition may result in continuous reproduction in *Liophis miliaris* from this region, as evidenced by the year-round presence of vitellogenic follicles. However, even continuous cycles show reproductive peaks in the warmer and rainier periods (*cf.* Seigel & Ford, 1987; Pizzatto & Marques, 2002). In this region, the small increase in temperature from October to March can cause a reproductive peak at the end of the year (suggested in Fig. 3) and a recruitment peak in January (see Fig. 4). There is no evidence of multiple clutch production in *L. miliaris* from the NCAF (Pizzatto & Marques, 2006), thus a continuous cycle within this population may be due to asynchronous reproduction (*cf.* Seigel & Ford, 1987).

In the remaining populations, where climatic variation is more marked, reproduction seems to be seasonal with oviposition taking place during the rainy season. In SCAF and NIAF, temperature variation is tenuous but with a marked dry season (from April to September). *Liophis miliaris* feeds upon aquatic prey in aquatic habitats (Marques & Souza, 1993; Marques & Sazima, 2004) and in southeastern Atlantic forest (SCAF and NIAF) a decrease in the number of temporary ponds in the dry season restricts prey availability (Marques *et al.*, 2001). Thus, rainfall may be the principle factor that restricts the reproductive cycle in female *L. miliaris* in these regions. Reproductive seasonality has also been observed in other snakes and lizards from tropical areas where monthly temperature variation is minimal but seasonality of rainfall is marked   (*cf.* Saint-Girons, 1982; James & Shine, 1985; Shine *et al.*, 1998; Brown & Shine, 2006). On the other hand rainfall is homogeneous throughout year in SIAF but the reproductive cycles of snakes are seasonal. Female abundance is related to minimum temperature in this area. *Liophis miliaris* tends to remain coiled and inactive at temperatures between 12 and 18°C (Abe, 1977) and, in SIAF, mean temperatures are about 13°C from June to August and minimum temperatures are under 10°C. The reproductive season in SIAF starts only in October when minimum temperatures are close to 15 - 20° C and extends until February, while in the other seasonal areas it is from late August to February. Thus, in SIAF the beginning and end of the reproductive period may be delayed because of a delay in the arrival of warmer weather compared to SCAF and NIAF. This pattern is similar to those described for other tropical (Madsen & Shine, 1996) and temperate snakes (Naulleau *et al.*, 1998).

Reproductive seasonality in *Liophis miliaris* from SCAF, NIAF and SIAF is probably due to reproductive synchrony within these populations, and multiple clutches can also occur (Pizzatto & Marques, 2006). Egg-laying during warmer and rainier periods tend to benefit egg incubation during more favourable climatic conditions for embryonic development (Vinegar, 1977), diminishing egg incubation time, increasing hatching success, and generating larger neonates (Qualls & Shine, 1998; Ji & Dou, 2001; Shine & Elphick 2001).

Multiple clutches in captive females maintained in the absence of males is indicative of long-term sperm storage (from a particular reproductive season to the next), which in some colubrids has been shown to occur in the female infundibulum (see Fox, 1956; Halpert *et al.*, 1982; Aldridge, 1992; Pizzatto & Marques, 2002).

TESTICULAR CYCLES

The relationship between testis volume and body size is a good indication of part of male reproductive effort (Begon *et al.*, 1990) and it also permits the description of testicular cycles in snakes (Volsøe, 1944; James & Shine, 1985; Pleguezuelos & Feriche, 1999; Shine *et al.*, 1999). Continuous sperm production is suggested by at least three factors. The first one is the absence of variation in residual testis volume in all populations. The second is the absence of variation in residual diameter of the deferent ducts, suggesting that sperm are not stored by males. In some snakes species, sperm storage by males in the deferent duct (which increases its diameter) generally occurs when spermatogenesis is seasonal (or continuous with a peak) and mating is dissociated from sperm production (Shine, 1977b; Almeida-Santos *et al.*, 2004). The third one is that climatic variation is not related to male abundance (and consequently male activity), in most populations, presumably it has little influence on sperm production. As mating is simultaneous with sperm production, male reproductive cycles may be classified as pre-nuptial, at least in NIAF and SCAF (see Saint-Girons, 1982; Seigel & Ford, 1987). In any case, small variations in sperm production are only detected by histological analyses.

ACTIVITY

In the areas where reproductive cycles are seasonal, snake activity can be directly related to both reproduction and feeding. Female abundance (and consequently activity – see Marques *et al.*, 2001) increases from October to December, when they are reproductively active. As demonstrated for other species, reproductive females often spend more time basking and are slower in their movements than non-reproductive ones (Shine, 1979; 1980b; Seigel *et al.*, 1987). Females also become more active during egg-laying periods as they search for suitable oviposition sites. In addition, female *Liophis miliaris* did not stop feeding in the early stage of reproduction (*i.e.* secondary vitellogenesis), when they need energy to invest in egg production. A high percentage of females with prey in the stomach had fol-

licles in secondary vitellogenesis. This suggests that females must increase feeding activity during early reproductive time. Thus, females may be more commonly collected during the reproductive season both due to reproductive and feeding activity.

Activity in *Liophis miliaris* may be related to certain climatic factors (see Gibbons & Semlitsch, 1987). In SIAF, temperature decrease is more pronounced in autumn-winter than in the other areas and probably causes a decrease in female activity, reflected in the number of individuals collected. Even in NCAF where climate is more homogeneous minimum temperature has a significant influence on female activity. In the other areas there are no obvious relationships between the number of snakes and the environmental variables measured.

In southern areas, a decrease in male activity occurs only in the middle of the coolest and driest season (July to September) and the number of males from April to June (early cold/dry season) did not differ from the expected distribution. This probably happens because during this period males are engaged in searching for females (*cf.* Shine, 1980*a*) and do not limit their activity even with the decrease in temperature/rainfall.

In conclusion, female reproductive pattern in *Liophis miliaris* from Brazil differs among populations, mainly between those from northward (NCAF) to southward (SCAF, NIAF and SIAF) and these differences must be primarily due to the influence of temperature.

ACKNOWLEDGMENTS

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