ZOOLOGICAL RESEARCH

Exploring the reproductive ecology of the tropical semifossorial snake *Ninia atrata*

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ABSTRACT

Based on histological analyses and field studies, this research describes the reproductive ecology of a population of *Ninia atrata* snakes inhabiting an oil palm plantation. Furthermore, through a multivariate approach, we explored the main drivers of reproductive output in *N. atrata*. Results showed that prey abundance and food intake were crucial variables contributing to reproductive output. Multiple linear regression models showed that neonates had high sensitivity (R^2=55.29%) to extreme changes in climate, which was strongly related to slug and snail abundance variability and microhabitat quality. Reproductive cycles were markedly different between the sexes, being continuous in males and cyclical in females. Despite this variation, reproductive cycles at the population level were seasonal semi-synchronous. Constant recruitment of neonates all year, multiple clutches, high mating frequency, and continuous sperm production characterized the reproductive phenology of *N. atrata*. In addition, a significant number of previtellogenic females presented oviductal sperm as well as uterine scars, suggesting a high precocity in the species. The main drivers of reproductive output also differed between the sexes. In females, clutch size and secondary follicle variability were highly related to stomach bolus volume, fat body area, and body mass. In males, height of piles of palm leaves and body mass, rather than intrinsic reproductive traits, were the main drivers of sperm production. Nevertheless, in both cases, the relationship between body mass, prey abundance, and food intake suggests that *N. atrata* follows the income breeding strategy to compensate for reproductive costs and to maximize fitness.

Keywords: Continuous male reproduction; Clutch mass; Income breeding; Iteroparity; Spermatogenesis; Oogenesis; Reproductiveeffort; El Niño-Southern Oscillation (ENSO)

INTRODUCTION

Since the early efforts of Fitch (1970) to elucidate the reproductive cycles of tropical reptiles, research on the reproductive phenology of tropical snakes has increased in response to the historical disparity between temperate and tropical zone studies (Mathies, 2011). In particular, researchers in the South American tropics of Brazil and

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Argentina have expanded our knowledge about the phenology and reproductive traits of more than 10 elapid species (Almeida-Santos et al., 1998, 2006; Ávila et al, 2010; Valdujo et al., 2002), 10 viper species (Almeida-Santos et al., 1999, 2006; Almeida- Santos & Salomao, 2002; Hartmann et al., 2004; Janeiro- Cinquini, 2004; Leão et al., 2014; Marques et al., 2013; Monteiro et al., 2006; Nogueira et al., 2003), nine boine species (Bretona & Chiaraviglio, 2003; Chiaraviglio, 2006; Miranda et al., 2017; Pizzatto, 2005; Pizzatto & Marques, 2007; Rivas et al., 2007), and 26 colubrid species (Alencar et al., 2012; Ávila et al., 2006; Balestrin & Di-Bernardo, 2005; Bizerra et al., 2005; Braz et al., 2014; da Costa-Prudente et al., 2014; Dos Santos-Acosta et al., 2006; Gaiarsa et al., 2013; Goldberg, 2004b, 2006 Gomes & Marques, 2012; Gualdrón-Durán et al., 2019; Hartmann et al., 2002; Leite et al., 2009; López & Giraudo, 2008; Marques, 1996; Marques et al., 2009; Marques & Puorto, 1998; Pizzatto et al., 2008; Pizzatto & Marques, 2002; Scartozzoni et al., 2009; Silva & Vadez, 1989; Vitt, 1996).

The above contributions have shown that the reproductive biology of tropical snakes diverges from the uniformly seasonal and highly synchronous patterns seen in species from temperate zones. Tropical snakes exhibit both seasonal and aseasonal reproductive cycles. However, aseasonal reproduction is not the rule in the tropics, in fact, truly aseasonal (continuous) reproduction by tropical females is rare (Brown & Shine, 2006b; Saint Girons & Pfeffer, 1972; Shine, 2003). Tropical snake reproduction includes multiple phenologies and high variability among populations, as well as within individuals of the same population (Mathies, 2011; Pizzatto et al., 2008). Most species also have broad seasonal reproductive schedules and exhibit intersexual divergence in reproductive cycles (e.g., *Naja*, *Bungarus*, *Calliophis*, Pizzatto et al., 2008; Saint Girons & Pfeffer, 1972).

Despite the enormous efforts to understand the reproductive biology of snakes, fewer studies have been conducted on tropical fossorial and semifossorial snakes than on terrestrial and arboreal snakes (Braz et al., 2014). This is due to the cryptic behavior, secretive microhabitats, and lower encounter rates of fossorial and semifossorial snakes, which makes them an elusive research object. Therefore, their natural history and ecology remain poorly understood, which limits our assessment of their reproductive seasonality, energy acquisition timescale, reproductive expenditure, and sexual maturation age or size. In Colombia, the semifossorial snake *Atractus marthae* (Meneses-Pelayo & Passos, 2019) has been the only snake species with a detailed reproductive study. *Atractus marthae* populations inhabit the cloud forests (>2 400 m a.s.l.) of the northeastern Andes of Colombia, with females reported to have an asynchronous reproductive stage, aseasonal and discontinuous reproductive cycle, and single clutch per year, whereas males present spermatozoa in testes and ducts, as well as hypertrophied of the sexual segment of male kidney (SSK) throughout the year (Guladrón-Durán et al., 2019). However, given the dearth of reproductive information among Colombian snakes, comparison between the reproductive ecology of snakes from highlands and lowlands is difficult, limiting our understanding of the general reproductive patterns of snakes in the tropical Andes.

Ninia atrata (Hallowell, 1845) is a semifossorial tropical snake widely distributed in South America. It ranges from Western Panama, Colombia, Ecuador, Venezuela to Trinidad and Tobago (Angarita-Sierra, 2009, 2014, 2015; Ingrasci, 2011; McCranie & Wilson, 1995; Medina-Rangel, 2015; Mesa-Joya, 2015; Rivas et al., 2012). Despite its broad distribution and high abundance in disturbed or transformed habitats, its reproductive biology has been largely ignored (Angarita-Sierra, 2015; Lynch, 2015). Currently, details on clutch size and birth-size in northern South American populations have only been reported as anecdotal observations (Lancini, 1979; Natera-Mumaw et al., 2015; Roze, 1966). Silva & Valdez (1989) reported a hatchling period from June to September in populations located on the northern hills of Caracas, Venezuela.

Herein, we explored the reproductive biology of *N. atrata* in order to improve our understanding of the reproductive features of semifossorial tropical snakes. Our study consisted of three main goals: (1) To provide a detailed description of female and male annual reproductive cycles, minimum size at sexual maturity, mating frequency, recruitment, clutch size, and egg features. (2) To analyze the relationship between reproductive traits of *N. atrata*, prey abundance, and food intake, as well as variability during extreme El Niño-Southern Oscillation (ENSO) climatic events. (3) To explore the main drivers of reproductive output of the population under study.

MATERIALS AND METHODS

Study area and climate variability

We studied *N. atrata* snakes inhabiting the oil palm plantation (*Elaeis guineensis* Jacq. 1897) of Palmasol S. A., located at Vereda La Castañeda, the municipality of San Martin, Department of Meta, Colombia (N3° 31', W73° 32'; Figure 1). This locality has a monomodal climate (rainy season from April to November and dry season from December to March) with an annual rainfall of the 3 070 mm and high temperatures year-round (>26 °C). According to the Colombian Institute of Hydrology, Meteorology, and Environmental Studies (IDEAM Spanish acronym), the El Niño ENSO recorded between 2016 and 2017 was the strongest of the last 20 years (IDEAM, 2016). Therefore, climatic variability was categorized as good and bad climatic years, where good years represent the sampling period from August 2014 to December 2015 without ENSO effects, and bad years represent the sampling period from January 2016 to April 2017 under ENSO effects.

We monitored the temperature and relative humidity of microhabitats using four thermo-hygrometers (EBI20-TH1 Ebro®). These devices were placed over three consecutive days per sampling visit, with three at the base of different piles of palm leaves and one at the base of an epiphytic cushion. These sites were randomly selected. A fifth thermo-hygrometer was placed at a height of 1.5 m on a randomly

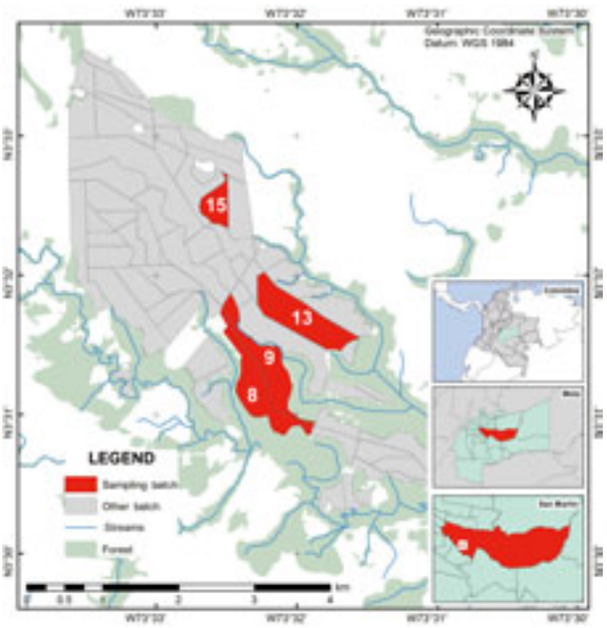

Figure 1 Study area

Oil palm plantation (*Elaeis guineensis* Jacq. 1897) of Palmasol S. A. Sampling batches 8, 9, 13, and 15 are in red

selected palm tree trunk. We configured the thermo-hygrometers to record temperature and relative humidity readings on a per minute basis, resulting in a total of 138 240 measurements and 2 304 h of sampling effort per thermo-hygrometer.

Sampling and data collection

Data were obtained during four plantation production batches (8, 9, 13, 15; Figure 1), in which piles of oil palm leaves are stacked following pruning. We sampled from August 2014 to June 2017, spending three days per sampling visit (n=32). No sampling was conducted in September 2014 or May 2015 due to logistic constraints.

Snake searching was conducted in a total of 4 563 palm leaf piles (microhabitat 1) and 304 epiphytic cushions attached to the base of palm trees (microhabitat 2) from 0730 h to 1730 h, totaling 1 189 h of sampling effort per researcher. Palm leaf piles and epiphytic cushions were searched by raking to a depth of 5–15 cm over a 3–5 min period, with all hand-captured prey items (e.g., snails, earthworms, slugs, and leeches) and snakes recorded. We used the height (cm) of the palm leaf piles and epiphytic cushions as surrogate estimators of microhabitat quality. This is because greater habitat height likely provided better refuge for the snakes due to lower microclimate variability, larger number of prey, and greater security against predators (Angarita-Sierra, 2015; Angarita-Sierra & Lozano-Daza, 2019; Lynch, 2015; Weatherhead & Madsen, 2006).

All caught snakes were measured (snout-vent length (SVL); tail length (TL)) using a measuring tape (±0.1 cm) and weighed (Mass) using a Pesola® dynamometer of 50 g (±0.1 g). We calculated the sexual size dimorphism index (SSD) following Gibbons & Lovich (1990) and determined the ratio between the SVL of the larger and smaller sex. The ratio is defined as positive when females are larger and negative when males are larger (with SSD=1.0 when SVL is equal between the two sexes). We recorded snake health (presence/ absence of tick, mycoses, or injuries) by external examination, as well as sex, umbilicus scars, and secondary sexual traits in males (development degree of chin tubercles). We also recorded the presence of food and reproductive condition by palpation and by contrast light on the snake body from the dorsal to ventral surface.

Snakes captured in batch 13 (n=275) were used in the mark-recapture experiments to test whether females could produce more than one clutch during the same reproductive season. Thus, these snakes were branded on their ventral scales following the procedures described by Dorcas & Willson (2009) using an Aaron Medical Change-A-Tip cautery unit Bovie® (Winne et al., 2006). We determined the sex of the branded snakes by inserting a blunt probe through the cloaca orifice following procedures described by Blanchard & Finster (1933). Afterward, all branded individuals were released at the same place in which they were captured.

We transported gravid females and eggs found in the field to the lab in individual terraria. The females were provided with food and water *ad libitum*. The captive environment was maintained to resemble the conditions of the oil palm plantation (photoperiod regime: 12 h light/12 h darkness; temperature: 26.21±0.61 °C and 65.64±3.52 h). Once females laid their eggs, we collected, measured, inspected, and monitored their temperature throughout incubation (26.21±0.61 °C; 65.64±3.52 h) using an infrared thermometer (CGHM-H13). Finally, when hatchlings were born, we measured, individually marked, and released them with their mothers at the place where she was captured.

Reproductive data acquisition

In total, 150 snakes from batches 8, 9, and 15 were euthanized with an injection of lidocaine 2% ($C_{14}H_{22}N_2O$) in the heart, then fixed with 10% formalin, and preserved in 70% ethanol. All specimens were deposited in the reptile collection of the Instituto de Ciencias Naturales (ICN) of the Universidad Nacional de Colombia. We determined sex of the collected snakes by abdominal dissection and direct gonad observation.

Afterward, the reproductive tracts were fixed in 10% buffered formalin and then used to make histological slides following Luna (1968) to determine reproductive stage, as well as spermatogenesis and oogenesis cycles. We registered microscopic reproductive traits following the procedures described by Krohmer et al., (2004), Balestrin & Di-Bernardo (2005), and Ramos-Pallares et al. (2015). Sperm abundance and time of spermatogenesis were based on counts (three replicates) in cross-sections of seminiferous tubules chosen at random using a light microscope under 40× magnification (Fox, 1952).

Based on digital pictures and ImageJ v1.52 software (Schneider et al., 2012), we obtained the following

macroscopic reproductive variables from sacrificed male and female snakes: size of hypertrophy SSK, testicular volume (mm^3), width of distal end of deferent duct (mm), oviductal width (mm), number and diameter of previtellogenic and vitellogenic follicles (mm), and width and length of oviductal eggs (mm).

We considered sexually mature males as the smallest (SVL) male having spermatozoa in their testes, and sexually mature females as the smallest female with vitellogenic follicles or oviductal eggs. We calculated relative fecundity (RF) and relative clutch mass (RCM) following Iverson (1987) and Seigel & Fitch (1984), respectively. We classified female reproductive condition as previtellogenic (only translucent tiny follicles), vitellogenic (yellowish yolky follicles), ovigerous (with oviductal eggs), or vitellogenic and ovigerous (with vitellogenic follicles and oviductal eggs simultaneously). We employed histological cuts to evaluate and validate the macroscopic state of the follicles, which allowed us to allocate an unbiased reproductive state for both males and females. Finally, we used the presence or absence of sperm in the oviducts and infundibulum as a surrogate estimator of mating season.

Statistical analysis

We assessed differences in the sex ratio among mature males and females each month using the *G*-test and Chi-square (χ^2) test. We compared size at sexual maturity between males and females using *t*- test and assessed the assumptions of normality and homoscedasticity based on Kolmogorov-Smirnov and Levene tests, respectively (Guisande-Gonzáles et al., 2014). We evaluated monthly intersexual variations (synchrony) in female and male reproductive stages, as well as time variation (seasonality) using the χ^2 test and *G*-test. Likewise, we employed one-way analysis of variance (ANOVA) to compare the oviduct distal width between female reproductive stages.

Based on multiple regression analyses of 150 collected snakes (females=79, males=71), we explored the main drivers of reproductive output of the *N. atrata* population under study. We first considered the following variables: SVL (Var 1), body mass (Var 2), primary follicle number (Var 3), secondary follicle number (Var 4), fat body area (Var 5), stomach volume (Var 6), height of piles of palm leaves (Var 9), testicular volume (Var 10), SSK width (Var 11), and width of distal end of deferent duct (Var 12). We then measured stomach bolus and fat bodies through digital pictures with ImageJ v1.52 (Schneider et al., 2012). We calculated fat body area (mm^2) as the sum of each small fat body area attached to the digestive and reproductive tract of each individual. We estimated testes and stomach bolus volume employing the ellipsoid formula (equation 1):

$$V = \frac{3}{4}\pi\left(\frac{w}{2}\right)^2\left(\frac{l}{2}\right) \quad (1)$$

where, V=testes volume estimated or stomach bolus, *w*=width, and *l*=length.

We next used clutch size and sperm count as dependent variables, with all remaining variables considered independent. Both dependent and independent variables were square roottransformed using the Tukey's staircase transformation method described by Erickson & Nosanchuk (1977).

We evaluated assumptions of normality, autocorrelation, and homoscedasticity using the Kolmogorov-Smirnov test, Durbin-Watson test, and Breusch-Pagan test, respectively. We tested multicollinearity between the previously named variables using the variance inflation factor (VIF) with a threshold of 10. To select the "best" regression model based on the variables evaluated, we employed the Akaike Information Criterion (AIC; Akaike, 1973).

We analyzed recruitment variability during ENSO events, employing neonatal abundance as the dependent variable and height of piles of palm leaves (Var 9) and prey abundance observed in microhabitats (snails, slugs, earthworms, leeches; Angarita-Sierra & Lozano-Daza, 2019) as independent variables.

Finally, to estimate the contribution of all independent variables to the regression models, we employed a hierarchical partitioning method (Chevan & Sutherland, 1991). All statistical analyses were performed using Rwizard 3.0 (Guisande-Gonzáles et al., 2014) and the following R packages: StatR (Guisande-Gonzáles et al., 2014), hier. part (Walsh & MacNally, 2015), nortest (Gross, 2015), lmtest (Hothorn et al., 2017), and usdm (Naimi, 2015).

RESULTS

From all batches sampled, we caught a total of 425 specimens of *N. atrata* (males=209, females=216), with an SSD index of 0.16. The sex ratio showed no significant differences between months (χ^2=15.89, *P*=0.145, *n*=425), climate season (χ^2=2.30, *P*=0.1286, *n*= 425), or batches sampled (χ^2=0.93, *P*=0.61, *n*=425). In contrast, significant differences in snake abundance were observed between good and bad years (χ^2=176.15, *P*<0.0001, *n*=425; Figure 2). Health condition was recorded as a potential variable affecting reproductive output. However, through the whole sampling period, only 11 specimens (2.6%) suffered poor condition (ticks *n*=1; mycoses *n*=8; injury=2). Therefore, these variables were excluded from analyses and we considered the *N. atrata* population under study to be healthy.

Female reproductive cycle and activity

According to the macroscopic reproductive traits observed in 79 *N. atrata* females, the smallest female with vitellogenic follicles, indicating sexual maturity, was 270 mm SVL. All females larger than 270 mm SVL (48%) were in some stage of reproduction (Table 1). Females were asynchronous in reproductive stage between months or climate years (χ^2 months=27.374, *P*=0.44; χ^2 climate years=3.05, *P*= 0.38). All reproductive stages were observed throughout the year. Oviduct width exhibited significant differences among female reproductive stages (ANOVA *F*=13.7, *P* <0.000 1), with the oviduct being less wide in previtellogenic females than the

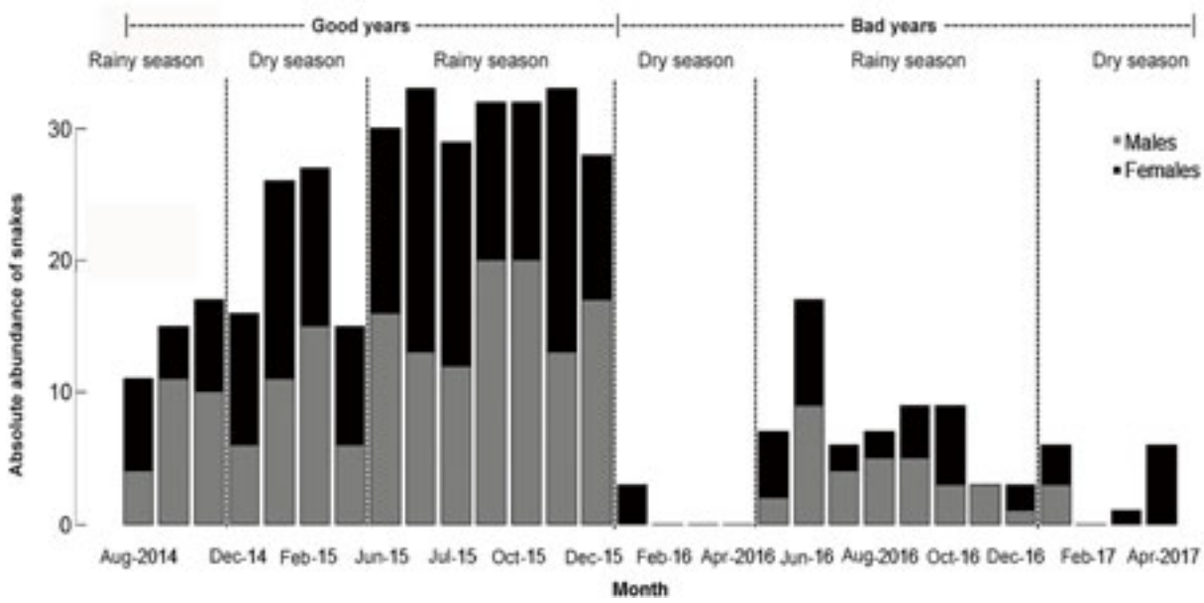

Figure 2 Abundance and population sex ratio of *Ninia atrata* through sampling period (2014−2017)

Table 1 Mean values of female reproductive features

Female reproductive stage	SVL (mm)	Mass (g)	Oviduct length (mm)	Sperm in oviduct	Uterine scar	No. primary follicles	No. secondary follicles	No. eggs
Previtellogenic	205.05 (120−246) *n*=38	4.94 (1.08−9.42) *n*=34	4.00 (0.91−9.75) *n*=38	Present (40%) Absent (60%) *n*=43	Present (15%) Absent (85%) *n*=38	5.71 (1−10) *n*=38	Absent	Absent
Vitellogenic	313.57 (270−353) *n*=14	12.01 (8.33−17.09) *n*=14	9.19 (2.59−16.85) *n*=14	Present (87.5%) Absent (12.5%) *n*=16	Present (20%) Absent (80%) *n*=10	7.21 (3−10) *n*=14	2.36 (1−5) *n*=10	Absent
Ovigerous	335.08 (278−370) *n*=12	15.88 (13.33−19.8) *n*=11	9.06 (4.10−13.49) *n*=12	Present (83.3%) Absent (16.7%) *n*=12	Present (100%) *n*=12	8.66 (5−13) *n*=12	Absent	3.25 (2−4) *n*=12
Vitellogenic and ovigerous	315.68 (320−370) *n*=5	15.02 (12.35−19.1) *n*=5	10.13 (5.19−14.4) *n*=5	Present (100%) *n*=7	Present (100%) *n*=5	10 (7−14) *n*=5	1.8 (1−3) *n*=5	2.8 (2−4) *n*=5

remaining reproductive stages. However, differences in oviduct width among females at ovigerous, vitellogenic, and vitellogenic-ovigerous stages were not significant (ANOVA F= 0.14, P= 0.86). Thus, an oviduct width larger than 6.14±3.85 mm indicated sexual maturity. Nonetheless, a high degree of oviduct width overlap was observed among female reproductive stages, suggesting that this macroscopic character is not an accurate predictor of sexual maturity (Figure 3).

In the 79 adult females examined, primary follicles were present throughout the year. However, in mid-June, most follicles began to enlarge, reaching a maximum in November to January until the beginning of the dry season (Figure 4A). Likewise, the greatest abundance of primary follicles was observed in January to March and September to November. Consequently, secondary follicles were only observed from April to November (rainy season), with abundance and size increasing gradually, reaching the greatest size in the October to November period (Figure 4B).

We observed mating signals at all female reproductive stages. However, the number of immature females exhibiting mating signals was significantly lower than that of mature females (χ^2=20.14, P<0.001, n= 79). As expected, the frequency of uterine scars was significantly higher in females at ovigerous and vitellogenic stages (χ^2=44.32, P<0.001, n=79). Notwithstanding, uterine scars also were observed in six previtellogenic females, four of which had sperm inside the infundibulum and two of which had sperm inside the oviduct. Mature females had sperm inside their infundibulum or oviduct almost the entire year (except December), indicating that copulation is continuous even for females not ready to mate. We observed eggs throughout the year, though the greatest

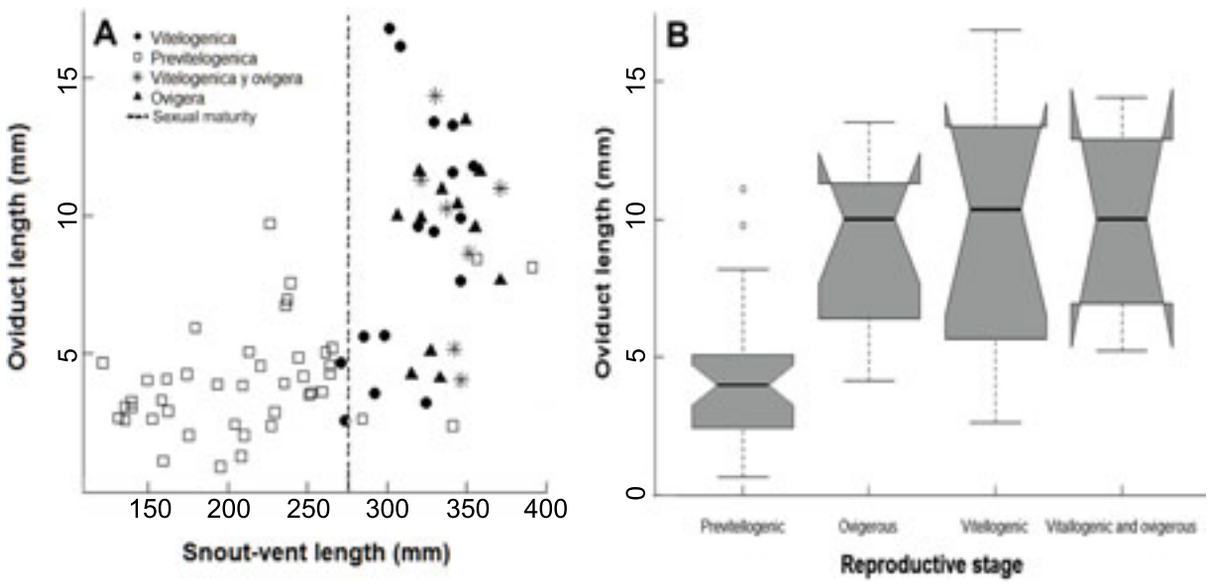

Figure 3 Variation in oviduct width

A: Scatterplot depicting relationship between snout-vent length (SVL) and distended degree of oviduct (mm). B: ANOVA showing oviduct length differences between female reproductive stages

abundance was recorded in September to December (Figure 4C). Based on the incubation of three clutches oviposited by three gravid females, the estimated birth-time was 108±1.41 d/egg. Clutch size ranged from 1–4 eggs (2.44±1.02, n= 34). We recaptured two female snakes who produced eggs twice in the same reproductive season (first capture in July and recapture in November). Also, during the dissection of snakes collected in August to November from batches 8 and 9, we recorded five females with vitellogenic follicles and oviductal eggs simultaneously, indicating that females could produce more than one clutch per reproductive season.

Neonates were also observed throughout most of the year (except July), with three abundance peaks during the recruitment season. The greatest recruitment peak occurred during the early dry season (January–February). A second moderate recruitment peak was observed in September, and a third pronounced recruitment peak occurred at the end of the rainy season (November). The greatest dearth occurred during the first half of the rainy season (June–July; Figure 4D). Based on the birth of eight neonates, birth-size was estimated to be 114.63±10.69 mm SVL and 1.91±0.74 g of body mass. Likewise, RCM and RF were highly variable and ranged from 4.32% to 8.54% (7.21±1.14%, n= 16) and 2.72% to 12.74% (7.28±3.01%, n=34), respectively.

We observed a remarkable decrease in neonates between good and bad climate years. Based on the presence or absence of the ENSO, the number of neonates declined significantly, from 57 in good years to four in bad years (10.557, P=0.001, n= 61). Despite this, we observed a clear synchronization between recruitment peaks and prey abundance in years without ENSO effects. Increased snail and slug abundance coincided with increased neonate abundance over the same time period (Figure 5). This relationship was confirmed by multiple regression analysis, with neonate abundance being strongly correlated with slug and snail abundance, but not with other prey (R^2=0.46, P= 0.032; Figure 6). Nonetheless, when the height of piles of palm leaves was included in the analysis, it provided a better model, explaining 55.29% (P=0.011) of the neonate abundance variability observed (Table 2).

Male reproductive activity

Ninia atrata males exhibited early sexual activity. The smallest male with sperm in their testes, indicating sexual maturity, was 145 mm SVL, and the largest male without sperm was 212 mm SVL. These males represent the extreme body-size limits of sexual maturity. However, 98.60% (n=71) of males larger than 187 mm SVL had sperm in their testes and deferent duct. The smallest male with metamorphosing spermatocytes was 137 mm SVL.

Based on macroscopic examination of the male gonads, testes showed noticeable size variation throughout the year. Testicular size gradually changed, decreasing from February to August and increasing from August to November. Maximum volume was attained in April (beginning of rainy season) and October-November (end of rainy season) and was 136.4% greater than the minimum testicular volume observed in August (mid rainy season; Figure 7A). Despite a lack of mature male samples in December, January, and March, our data suggests that testicular volume declines at the beginning of the dry season but increases mid-way through.

Likewise, macroscopic sexual features such as SSK and distal end of deferent duct exhibited a similar monthly

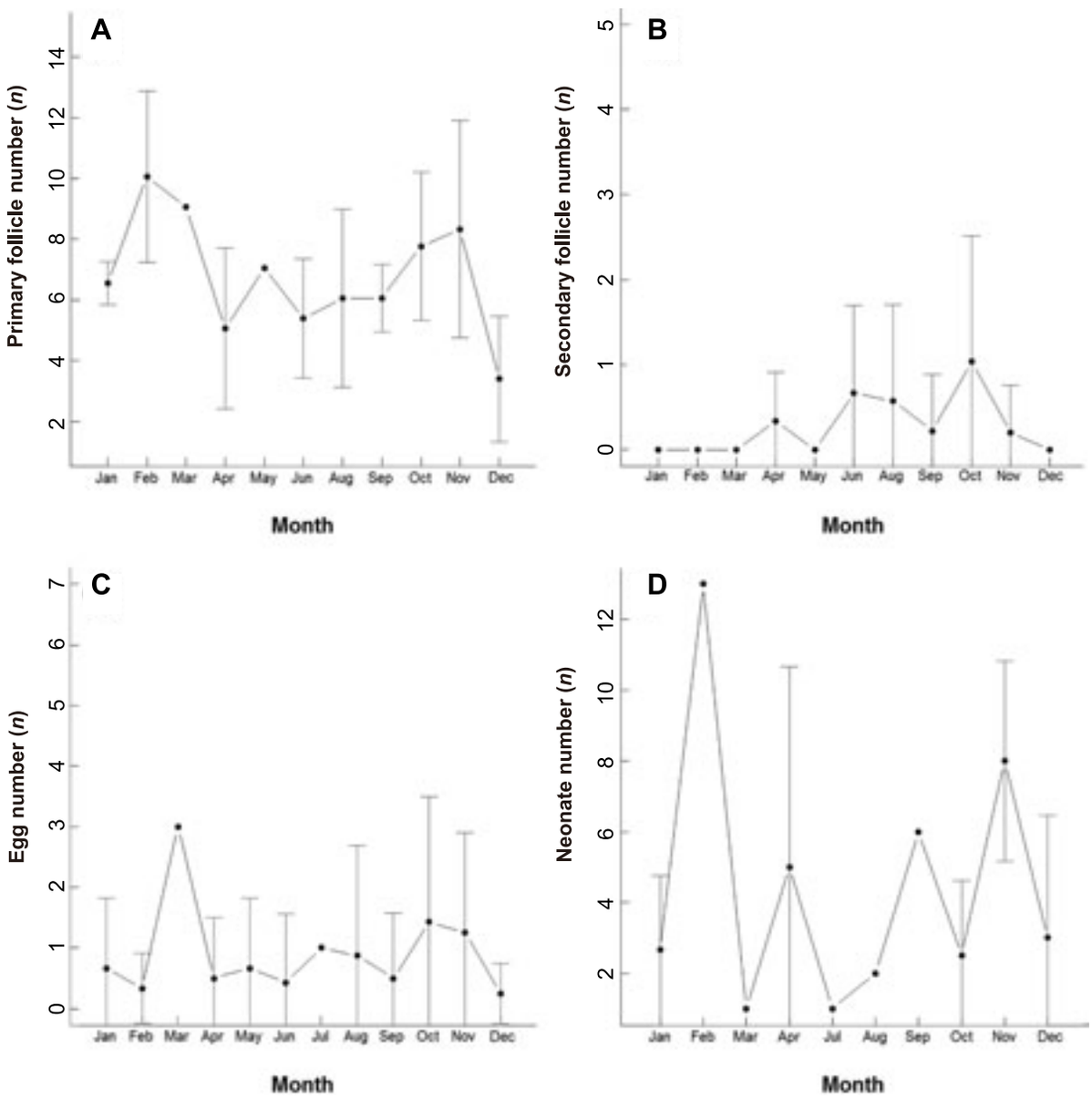

Figure 4 Monthly variability of female reproductive traits and neonates

Black dots represent means, black bars represent standard deviation. A: Absolute values of primary follicles. B: Absolute values of secondary follicles. C: Absolute values of eggs found during sampling period and in dissected reproductive tracts. D: Absolute values of neonates during sampling period

variability pattern as observed for testicular volume (Figure 7C–D). Indeed, monthly variability of these traits was closely related to testis size (R_{SSK}^2 =0.68, P<0.000 1, n=71; $R_{\text{deferent duct}}^2$= 0.45, P <0.000 1, n= 71). In contrast, sperm production was not correlated with monthly variability in macroscopic male sexual features (R_{SSK}^2 =0.031, P=0.15, n=69; $R_{\text{Testis volume}}^2$=0.028, P=0.18, n=71; $R_{\text{deferent duct}}^2$=0.033, P=0.14, n= 71), indicating that testicular volume, SSK hypertrophy, and deferent duct width are not concordant with spermatogenic activity. Sperm production was present for most of the year, including the dry and rainy seasons. Specifically, production increased gradually from April to November and reached a maximum in July–August (mid rainy season) without significant decline once production began (Figure 7B).

Conversely, significant differences in the size of males with either weak or prominent chin tubercles were found (ANOVA F=37.28, P<0.000 1, n=71). The weak condition was generally associated with males within the SVL range of 135–241 mm (168.16±2.02, n= 12), although two larger males (SVL=276

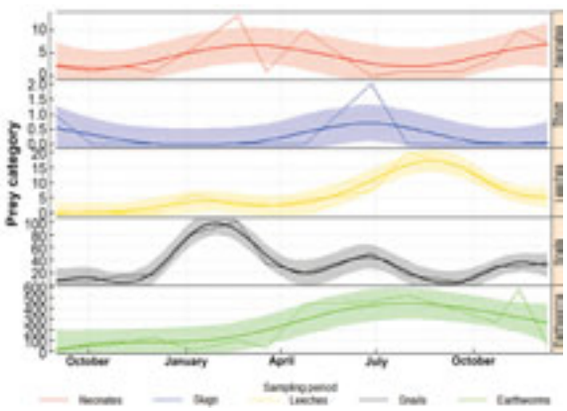

Figure 5 Temporal prey abundance variation and neonates of *Ninia atrata* during years without ENSO effects (2014−2015)

Scale of Y-axis represents abundance values. Shaded area represents 95% confidence intervals. Zigzag lines represent abundance values at each sampling period

and 280) exhibited this condition even though they had reached the minimum size of sexual maturity. In contrast, the prominent condition was generally associated with males within the SVL range of 183–354 mm, in accordance with the results that 98.60% (n=71) of males of this size have sperm in their testes and deferent duct. Despite this, three smaller-sized males (SVL=146, 154, and 175) also had prominent chin tubercles. In fact, the SVL ranges of chin tubercle condition showed a high degree of overlap (46.53%), indicating that this secondary sexual character may not be an accurate predictor of male reproductive stage.

Reproductive cycles and mating

We found notable differences in the reproductive cycles between the sexes. First, males showed a continuous cyclical pattern, in which spermatogenesis, gonads, and SSK were active throughout the year. Although they did show reduced activity during the dry and mid rainy seasons, they never displayed total regression or quiescence. In contrast, females showed a cyclical pattern in which oogenesis, gonads, and accessory organs become inactive or absent during the dry season. Second, size at sexual maturity was significantly different between the sexes (t=9.54, P<0.000 1, n=150), with males and females attaining sexual maturity at 56% and 86% of mean adult SVL, respectively. Finally, higher sperm and vitellogenic follicle production were not synchronized. While maximum sperm abundance occurred from July to August (mid rainy season), maximum vitellogenic follicle abundance occurred from October to November (end of rainy season).

Despite the divergence in reproductive cycles between sexes, sperm production and follicle maturation patterns indicated that the reproductive cycle was seasonal at the population level. Both sexes presented a synchronized increase in reproductive output through the rainy season, with highest abundance from June to November. Even though no mating behaviors were observed among individuals of *N.*

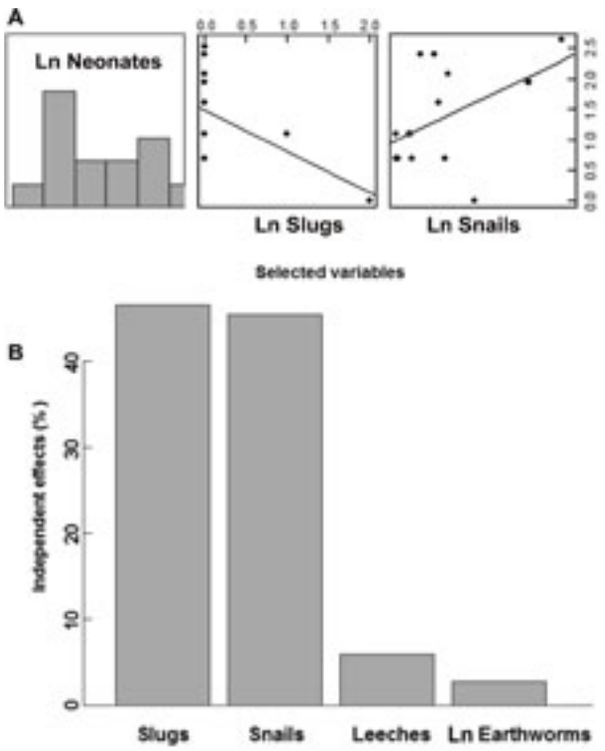

Figure 6 Multiple regression models related to neonate and prey abundances

Plots depicting linear regressions between neonate abundance (A) and prey abundance (B) Relative contributions of variables. Ln Neonates: Logarithm-transformed neonate abundance. Ln Earthworms: Logarithm-transformed earthworm abundance Ln Snails: Logarithm-transformed snail abundance. Ln Slugs: Logarithm-transformed slug abundance

atrata, the high frequencies of oviductal sperm, as well as sperm production and follicle maturation, suggest two main mating pulses, one at the beginning of the rainy season (April) and one at the end of the rainy season (October–November). However, mating signals were present all year, including the dry season (Figure 8).

The seasonal reproductive cycle of *N. atrata* was closely correlated with the strong climate seasonality of the study area, as well as prey and hatchling abundance. For instance, the greatest recruitment peak occurred in the mid dry season, which coincided with the increase in snail abundance and greatest production of primary follicles, whereas the greatest recruitment dearth was observed in the mid rainy season, which coincided with the decline in snail abundance and maximum sperm production (Figures 7–8).

Main drivers of reproductive output in *Ninia atrata*

The main drivers of reproductive output in *N. atrata* diverged between the sexes. In females, multiple regression analysis indicated that clutch size was strongly correlated with almost all reproductive traits evaluated (Table 2). However, among

Table 2 Multiple regression analysis models

Model	R^2	AIC	dAIC	*df*	Nor. test *P* value	Hom. test *P* value	Aut. test *P* value
Clutch size versus female reproductive drivers							
Clutch size vs. Var2+Var3+Var4	0.70	–90.62	0.0	1	0.07	0.35	0.63
Clutch size vs. Var2+Var3+Var4+Var5+Var6		–89.16	–1.46	1			
Clutch size vs. Var1+Var2+Var3+Var4+Var5+Var6		–87.28	–2.98	1			
Clutch size vs. Var1+Var2+Var3+Var4+Var5+Var6+Var9		–85.35	–5.39	1			
Secondary follicles versus female reproductive drivers							
Secondary follicles vs. Var2+Var5+Var6	0.42	11.18	0.0	1	0.17	0.36	0.87
Secondary follicles vs. Var2+Var5+Var6+Var9		11.69	0.51	1			
Secondary follicles vs. Var2+Var3+Var5+Var6+Var9		13.24	2.06	1			
Secondary follicles vs. Var1+Var2+Var3+Var5+Var6		15.21	4.03	1			
Sperm count versus male reproductive traits and environmental variables							
Sperm count vs. Var2+Var6+Var9	0.198	–166.21	0.0	1	0.07	0.34	0.2
Sperm count vs. Var1+Var2+Var6+Var9		–165.20	–1.01	1			
Sperm count vs. Var1+Var2+Var6+Var9+Var10		–163.32	–2.89	1			
Sperm count vs. Var1+Var2+Var6+Var9+Var10+Var11		–161.38	–4.83	1			
Neonates versus prey abundances							
Neonates vs. Slugs+Snails	0.46	–9.06	0.0	1	0.34	0.50	0.46
Neonates vs. Slugs+Snails+Leeches		–7.60	1.46	1			
Neonates vs. Slugs+Snails+Leeches+Earthworms		–6.41	2.65	1			
Neonates versus prey abundances and height of piles of palm leaves							
Neonates vs. Var9+Snails	0.55	–11.59	0.0	1	0.83	0.43	0.92
Neonates vs. Var9+Snails+ Slugs		–11.49	–0.10	1			
Neonates vs. Var9+Snails+ Slugs+Leeches		–10.55	–1.04	1			
Neonates vs. Var9+Snails+ Slugs+Leeches+Earthworms		–8.65	–2.94				

AIC: Akaike information criterion, employed to select"best model", was used to test whether environmental factors rather than intrinsic reproductive traits are main drivers of reproductive output. Var1: Snout-vent length, Var2: Body mass, Var3: Primary follicles number, Var4: Secondary follicles number, Var5: Fat body area, and Var6: Stomach bolus volume, Var9: Height of piles of palm leaves, Var10: Testicular volume, Var11: Width of sexual segment of kidney, and Var12: Distal width of deferent duct. R^2: Proportion of variance for reproductive output explained by microenvironment or reproductive intrinsic trait variables. Nor. test: Kolmogorov-Smirnov test for normality; Hom. test: Breusch -Pagan test for homoscedasticity; Aut. test: Durbin-Watson test for autocorrelation.

these variables, the "best model" was comprised of the number of primary and secondary follicles and body mass, which explained 70.63% (P<0.000 1) of clutch size variability. Given the importance of secondary follicles in clutch size, a second multiple regression analysis was carried out exploring the relationships among secondary follicles, maternal traits, and height of piles of palm leaves (Figure 9). As a result, secondary follicle variability was highly correlated with stomach bolus volume, fat body area, and body mass. These variables composed the "best model" and explained 44% (P= 0.003) of secondary follicle variability. Similarly, female SVL, but not the remaining variables, was significantly related to egg mass (F=7.64, P=0.014, n= 17), indicating that larger females produced heavier eggs.

In males, body mass, height of piles of palm leaves, and stomach volume, rather than intrinsic reproductive traits, showed the greatest contribution to sperm production (R^2= 0.198, P<0.001; Table 2). This result agrees with the discordance observed between sperm production and monthly variation in testicular volume and size of SSK (Figure 10).

DISCUSSION

In general, the reproductive ecology of *N. atrata* followed typical patterns reported in tropical snakes. First, early male maturation at a smaller size than females agrees with the common pattern among oviparous, small or median sized dipsadid species (Dos Santos-Acosta et al., 2006; Parker & Plummer, 1987; Pizzatto et al., 2008). Indeed, Goldberg (2004a) reported a similar maturation size for *N. maculata* (Peters, 1861) in which the smallest spermiogenic male was 179 mm SVL and the smallest vitellogenic female was 190 mm SVL.

Second, the reproductive cycles of both males and females were asynchronous, whereas the reproductive cycle at the population level was seasonal semi-synchronous, which agrees with the patterns observed in several tropical snakes with diverse phylogenetic histories, such as *Atractus marthae*

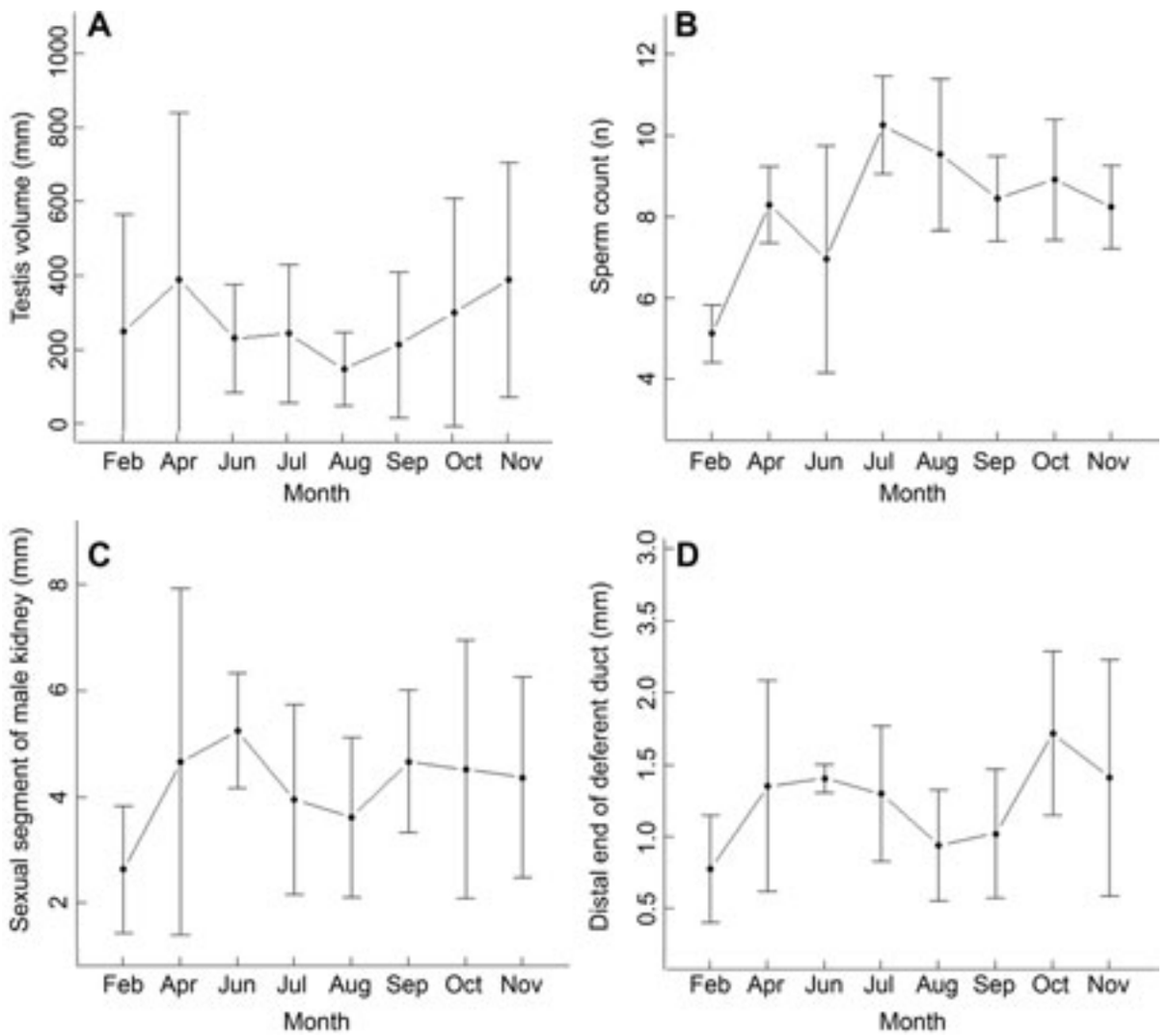

Figure 7 Monthly variability of male reproductive traits

Black dots represent means, black bars represent standard deviation. A: Absolute values of testis volume. B: Average values of sperm count per individual. C: Width of sexual segment of male kidney. D: Width of distal end of deferent duct

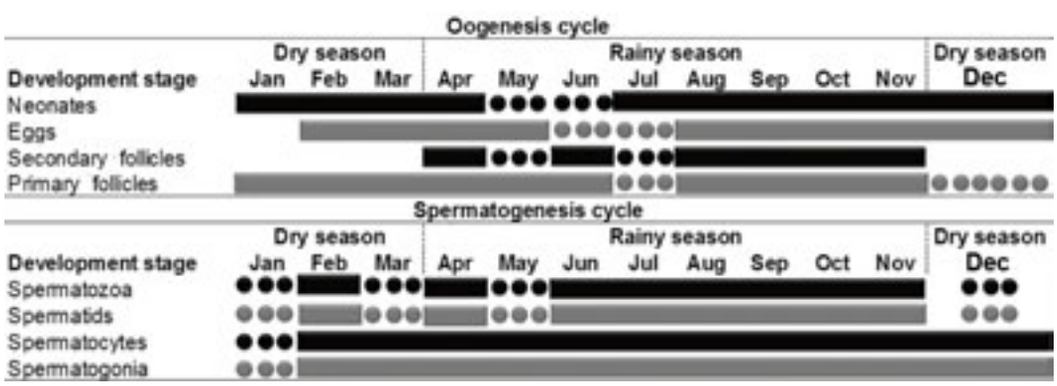

Figure 8 Oogenesis and spermatogenesis cycles in *Ninia atrata*

Females: 79, Males: 71. Boxes: Observed, dotted line: Inferred

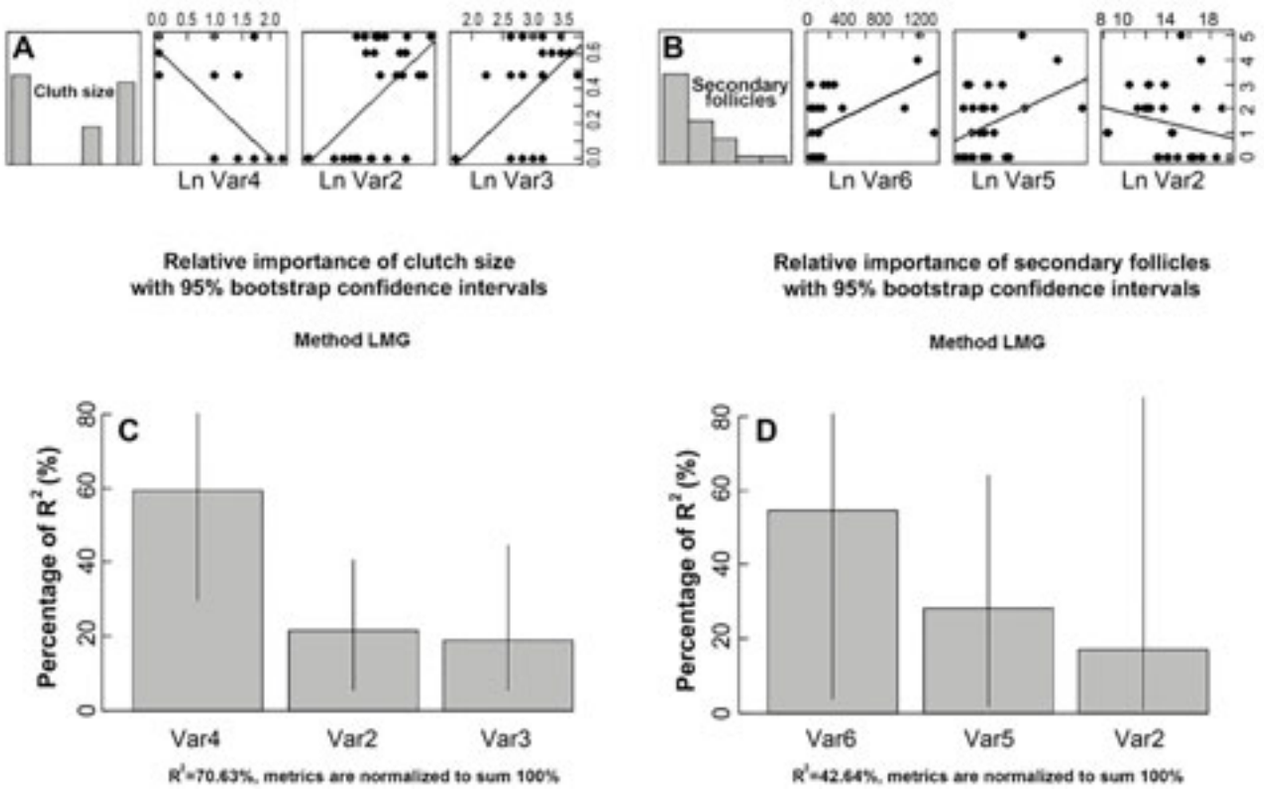

Figure 9 Multiple regression models related to clutch size and secondary follicles with environmental variables and intrinsic reproductive traits

A, B: Plots depicting linear regressions between clutch size and secondary follicles with variables, respectively. C, D: Relative contributions of variables composing"best regression model"for clutch size and secondary follicles, respectively. Var2: Body mass; Var3: Primary follicles number; Var4: Secondary follicles number; Var5: Fat body area; Var6: Stomach bolus volume

(Meneses-Pelayo & Passos, 2019), *Sibynomorphus* spp., (Schlegel, 1837), *Atractus reticulatus* (Boulenger, 1885), *Drymobius margaritiferus* (Schlegel, 1837), *Dipsas albifrons* (Sauvage, 1884), *Mastigodryas melanolomus* (Cope, 1868), *Micrurus lemniscatus* (Linnaeus, 1758), and others (Goldberg, 2006; Marques et al., 2013; Pizzatto et al., 2008).

Third, the presence of several vitellogenic-ovigerous females during the reproductive season, constant recruitment of neonates year-round, frequent mating evidence all year independent of season, and presence of previtellogenic females with sperm in their oviduct or infundibulum agrees with the patterns reported for numerous tropical snakes, such as *Erythrolamprus aesculapii* (Linnaeus, 1758), *Erithrolamprus bizona* (Jan, 1863), *Mastigodryas bifossatus* (Raddi, 1820), *Tropidonophis mairii* (Grey, 1841), and others, in which multiple clutches, high mating frequency, and continuous sperm production characterize their reproductive phenology (Brown & Shine, 2006a; Goldberg, 2004b, 2006 Marques, 1996). This suggests that immature females are willing or are forced by males to mate.

In contrast, the body size-fecundity relationship observed in *N. atrata* moves away from the expected correlation between SVL and reproductive output in tropical snakes (Miranda et al., 2017; Shine & Madsen, 1997). In *N. atrata*, SVL was shown to be a poor morphological predictor of fecundity. In particular, SVL was only significantly correlated with egg mass, presumably reflecting physical constraints on clutch volume (Shine, 1991). In contrast, body mass was shown to be a better morphological predictor for both sexes, as this trait was persistently selected in all regression models assessed. Male body mass had the strongest contribution (>50%) to sperm production. Similarly, female body mass in all regression models evaluated explained 20% of the reproductive output and occupied the second or third place of importance, after traits such as secondary follicle number, stomach bolus volume, or fat body area. Nonetheless, sperm production was poorly explained by the variables assessed (R^2=19.88%). Thus, future studies should clarify which microenvironment variables or intrinsic reproductive traits can determine the reproductive output observed in *N. atrata* males.

Food intake and prey abundance were crucial variables contributing to reproductive output in *N. atrata*. The high association of vitellogenic or ovigerous females with stomach content, as well as the great importance of stomach bolus volume as an explanatory variable of secondary follicle variability and sperm production, rather than fat bodies or

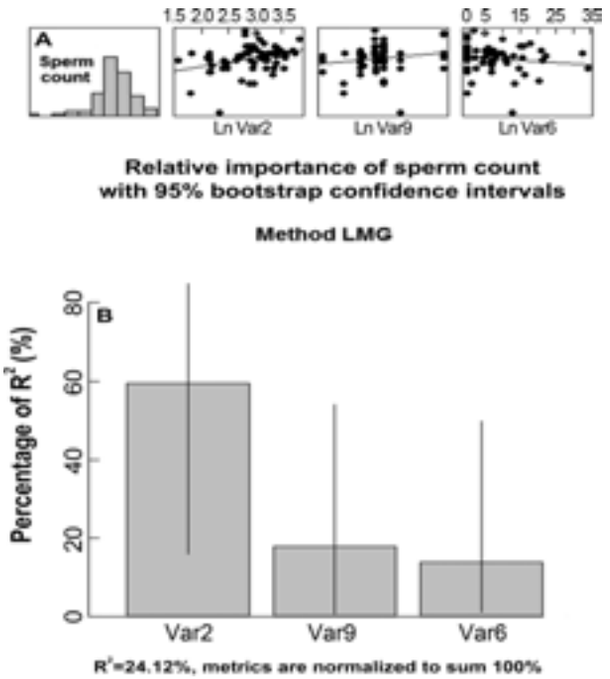

Figure 10 Multiple regression models related to sperm production with environmental variables and intrinsic reproductive traits

A: Plots depicting linear regression models between sperm count with variables. B: Relative contributions of variables composing "best regression model"for sperm count. Var2: Body mass; Var6: Stomach bolus volume; Var9: Height of piles of palm leaves

SVL, indicated a strong link between *N. atrata* reproductive output and food intake. In addition, the strong link observed between recruitment peaks and prey abundance is in agreement with the results of Shine (2003), who argued that even in the tropics, seasonal reproductive cycles would be favored because they reflect the variability of operative environmental factors, as well as the temporal shifts in reproductive trade-offs.

Similarly, the remarkable decrease in neonates and significant differences in snake abundance during ENSO events emphasized the high sensitivity of *N. atrata* to extreme climate changes. The variabilities of operative environmental factors such as height of palm leaf piles and prey availability (mainly snail abundance) were demonstrated to be the main drivers of *N. atrata* abundance variability (Angarita-Sierra & Lozano-Daza, 2019).

The above evidence suggests that *N. atrata* populations follow an income breeding strategy (Jönsson, 1997) in order to compensate for the demands of reproduction and to maximize fitness. This result agrees with the observed feeding patterns, i.e., largest females had significantly higher stomach bolus volumes than the largest males, intersexual dietary divergence, and notable disparity in food intake between the sexes (Angarita-Sierra & Lozano-Daza, 2019). Moreover, unlike tropical snakes that exhibit a seasonal shift in their reproductive strategies (from capital breeding to income breeding or vice versa, e. g., *Tropidonophis mairii* (Brown & Shine, 2006a)), *N. atrata* maintained the same reproductive strategy, despite the extreme climatic variability due to ENSO.

Income breeders are rare among ectotherms because of the high energetic costs associated with the muscle and organ maintenance needed to sustain their highly active mode of feeding (Bonnet et al., 1998). However, the *N. atrata* population in the oil palm plantation had a huge amount of prey available year-round, allowing them to reduce their energetic costs of acquisition without exceeding the energetic costs that would have to be expended in reproduction or other activities (Angarita-Sierra & Lozano-Daza, 2019). Hence, the income breeding strategy observed in this population could be strongly influenced by habitat. However, more empirical data are needed to elucidate whether reproductive strategy switches with habitat type or is a conservative life-history trait of the species.

Finally, macroscopic reproductive traits were shown to be an inaccurate proxy for reproductive activity in both sexes. In particular, testicular volume and SSK were not associated with sperm production. Likewise, oviduct distal width and presence of chin tubercles exhibited wide variance, which made it difficult for accurate maturity size determination. The accuracy of macroscopic reproductive traits as a proxy for reproductive cycle evaluation has been questioned previously (Braz et al., 2014; Mathies, 2011). In fact, it has been observed that histological analysis invalidates macroscopically determined maturity in fish, lizards, and fossorial snakes (Boretto & Ibargüengoytía, 2006; Braz et al., 2014; Fernández et al., 2017; Vitale et al., 2006). Nonetheless, this topic has been poorly explored in snake reproductive studies due to the historical and widespread use of macroscopic reproductive traits in comparisons between different studies (Pizzatto & Marques, 2006, 2007). Therefore, for future comparison between *N. atrata* populations or related taxa, we recommend employing histological assessment to avoid spurious results that could distort the relationships among reproductive cycles, environmental factors, and reproductive trade-offs.

SCIENTIFIC FIELD SURVEY PERMISSION INFORMATION

Permission for field surveys in the municipality of San Martin, Department of Meta, Colombia was granted by the National Environmental Licensing Authority (ANLA). Project approval (No. 27212 under resolution No. 0255 of 14 March 2014) was issued by the National Environmental Licensing Authority (ANLA).

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

T. A. S designed the study, collected data, conducted experiments in the field, analyzed data, and wrote the manuscript. C. A. L. H. collected data, conducted experiments in the field, and processed the data. T.A.S and C.A.

L. H. discussed and revised the manuscript. All authors read and approved the final version of the manuscript.

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