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RESEARCH ARTICLE

Ecological and reproductive aspects of *Aparasphenodon brunoi* (Anura: Hylidae) in an ombrophilous forest area of the Atlantic Rainforest Biome, Brazil

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ABSTRACT. Presented is the first information on the ecological and reproductive aspects of the treefrog, *Aparasphenodon brunoi* Miranda-Ribeiro, 1920, living in ombrophilous forest areas of the Atlantic Rainforest, Brazil. We recorded the species' daily activity and over the course of a year, population density during the year, microhabitat usage, diet, and some reproductive features (quantity, diameter and mean mass of oocytes, mean reproductive effort of female). Field sampling was conducted monthly from June 2015 to July 2016. Searches for treefrogs were systematic, using visual encounter surveys along 14 plots RAPELD long term research modules established in the forest. For each captured individual, we recorded the hour, microhabitat used, and perch height. The diet of the population was ascertained based on 15 individuals collected outside the study plot areas. Treefrogs used seven different types of microhabitats in the forest but the preferred microhabitats were tree-trunks and lianas. The amount of accumulated rainfall and air temperature interacted to explain the number of *A. brunoi* individuals active throughout the year. The reproductive strategy for females of this comparatively large arboreal frog in the ombrophilous forest is to produce clutches with a large number (900.8 ± 358.1) of relatively small-sized eggs. We conclude that in the ombrophious forest of the Vale Natural Reserve, *A. brunoi* is a nocturnal arboreal treefrog active throughout the year but activity increases during the wet season as a result of increased precipitation. In the forest, treefrogs tend to perch mainly on tree-trunks and lianas about 1 m above ground, where it feeds preferably on relatively large bodied arthropod prey. When living in the ombrophilous forest of the Atlantic rainforest, *A. brunoi* may change some features of its ecology (e.g. marked difference in the use of bromeliads) compared to when living in restinga habitats.

KEY WORDS. Casque-headed frog, ecological aspects, ecology, habitat use.

INTRODUCTION

Bruno's casque-headed frog, *Aparasphenodon brunoi* Miranda-Ribeiro, 1920, is endemic to the Brazilian Atlantic Rainforest (Haddad et al. 2013), occurring from the south of Bahia, southward to the state of São Paulo, along the states of Espírito Santo, Minas Gerais and Rio de Janeiro, and can be found mostly in restinga habitats of this Biome (Carvalho 1939, Feio et al. 1998, Argôlo 2000, Mollo Neto and Teixeira Jr 2012, Ruas et al. 2013, Haddad et al. 2013, Oliveira and Rocha 2014). Restinga is a typical environment of the Brazilian coast, which is characterized by sand dune formations (Rizzini 1997).

Although this species has been considered as decreasing in population size (Rocha et al. 2004), information regarding its ecology is still scarce. Along its distribution range, the relatively low information available providing aspects on its ecology comes from areas of restinga habitats (Teixeira et al. 2002, Mesquita et al. 2004, Sluys et al. 2004, Wogel et al. 2006, Haddad et al. 2013), with no information available on the ecology of the species found within ombrophilous forest environments of the Biome.

The occurrence of *A. brunoi* in forests is comparatively less frequent, with most records being composed of lists for the studied area (e.g. Feio et al. 1998, Silva-Soares et al. 2010, Almeida and Gasparini 2014). In one of the largest remnants of forests in the Atlantic Rainforest Biome, the Vale Natural Reserve (VNR) in the state of Espírito Santo in southeastern Brazil, *A. brunoi* was reported to occur (Almeida and Gasparini 2014) but with no local information regarding its ecology in this forested environment.

Recently, Jared et al. (2015) provided new biological information on this species by identifying highly toxic cutaneous secretions, and well-developed delivery mechanisms for both *A. brunoi* and *Corythomantis greeningi* Boulenger, 1896. The cutaneous secretions of these species present proteolytic and fibrinolytic action, as well as hyaluronidase that promotes the diffusion of toxins. These secretions are associated with a delivery mechanism which consists of using bony spines, located in the skull, that pierce the skin and inject the toxin into the predator (Jared et al. 2015). This unique defense mechanism makes *A. brunoi* a particularly important treefrog because it has a high toxicity, which is 25 times higher than that found in vipers of the genus *Bothrops*. Thus, aspects of their ecology are important for a better understanding of their biology in different environments.

Considering the known differences in the structural habitat among restinga and ombrophilous forest, we would expect that differences in aspects of the ecology of this frog could arise when living in the ombrophilous forest. In this context, we aimed to contribute information on such a unique species by analyzing the ecological and reproductive aspects of *A. brunoi* in the ombrophilous environment of the VNR over a one year period. We specifically addressed the following questions: i) What is the daily activity of *A. brunoi* and what is their activity throughout the year? ii) Which are the preferred microhabitats used by *A. brunoi* in the forest? iii) What is the vertical range of *A. brunoi* when perching in their habitat? iv) What prey composes the treefrog's diet and which prey items make up the majority of the diet? v) What is the overall morphometrics (mean quantity, diameter, and mass) of *A. brunoi* oocytes? vi) What is the average female reproductive effort for *A. brunoi*?

MATERIAL AND METHODS

The study was carried out in the Vale Natural Reserve (19°06'45"S, 40°03'03"W), located in Linhares and Jaguaré municipality, north of Espírito Santo, Southeastern Brazil. The reserve consists of approximately 23,500 ha and is one of the largest and most important remnants of the Atlantic Rainforest Biome (MMA 2000). The regional is tropical, rainy, and warm with mean annual rainfall of 1,214.6 mm and a mean annual temperature of 24.3 °C (Kierulff et al. 2014). The reserve is covered by a mosaic of habitats with four main vegetation types: Ombrophilous forest ("Tabuleiro"), a dense forest with trees reaching ca. 40 m height; the Sandy soil forest ("Mussununga forest"), which follows the cordons of sandy soils with trees of comparatively lower height and shrubs that allow most sunlight to reach the ground; the Permanent or Seasonally flooded forest (composed locally by swamps, and lowland and riparian forests) which are associated to water bodies that differ structurally; and the Natural grasslands ("Campos nativos"), which are open fields that emerge as enclaves in the forest, covered by herbaceous and shrubby vegetation forming thickets (Fig. 1, Peixoto et al. 2008). In this study, we sampled only the ombrophilous forest vegetation type.

Field sampling was done monthly from June 2015 through July 2016, including months from dry (April to September) and rainy (October to March) seasons in the area. Sampling was carried out during diurnal (11:00 am to 05:00 pm) and nocturnal (06:00 pm to 11 pm) periods in order to identify activity patterns of the treefrogs. Frog sampling was conducted in 14 plots using the RAPELD sampling method (Magnusson et al. 2005), distributed proportionally along four modules. This sampling method consisted of permanent and standardized plots of 250 m extensions each, following contour lines of the ground at a distance of 1 km between each.

Treefrogs were captured on transects along plots of the module using visual encounter and acoustic surveys (Crump and Scott 1994) simultaneously by two observers. Each plot was sampled five times but only once per month. Before sampling each plot, we measured air temperature (°C) and relative air humidity (%) using a thermohygrometer. During each transect, the plot was carefully inspected by the observers, looking for treefrogs in the leaf-litter, on trees, branches, bushes, fallen logs or other microhabitats when present. We were careful to record only active individuals (e.g. moving, foraging) to include in our activity analysis. All individuals were found and sampled within five meters from the center of each side of the plot (totaling a strip of 10 m wide). For each captured individual we recorded the hour, date, plot number, microhabitat used, and the height above ground (in cm) that the individual was perched when first sighted. At the end of each sampling period, air temperature and relative humidity were measured and recorded again. Data on daily rainfall (in mm) in the area of the Reserve were obtained from the meteorological station at the VNR.

We estimated treefrog density in the area (ind/ha), based on the area of each plot (250 m extension x 10 m wide = 2500 m^2), calculating the total searched area of plots [considering all plots transected/month x 2500 m^2 extension = total area searched (in m^2)] and divided the number of individuals of *A. brunoi* per month. Then, we calculated and compared mean treefrog density among the months of dry and rainy seasons using Student t-Test. We used Multiple Regression Analysis to evaluate the effects of temperature and accumulated rainfall of the sampling days for *A. brunoi* density and Simple Regression Analysis to evaluate the effect of humidity throughout the sampled days on *A. brunoi* density in the respective month of the sampling. We obtained the accumulated temperature and humidity in the sampled period from the averages of the values measured in the plots and obtained precipitation estimates of the sampled period from the sum of the accumulated precipitation that occurred during the sampling period. The analyses were conducted using the software R – 3.3.1.

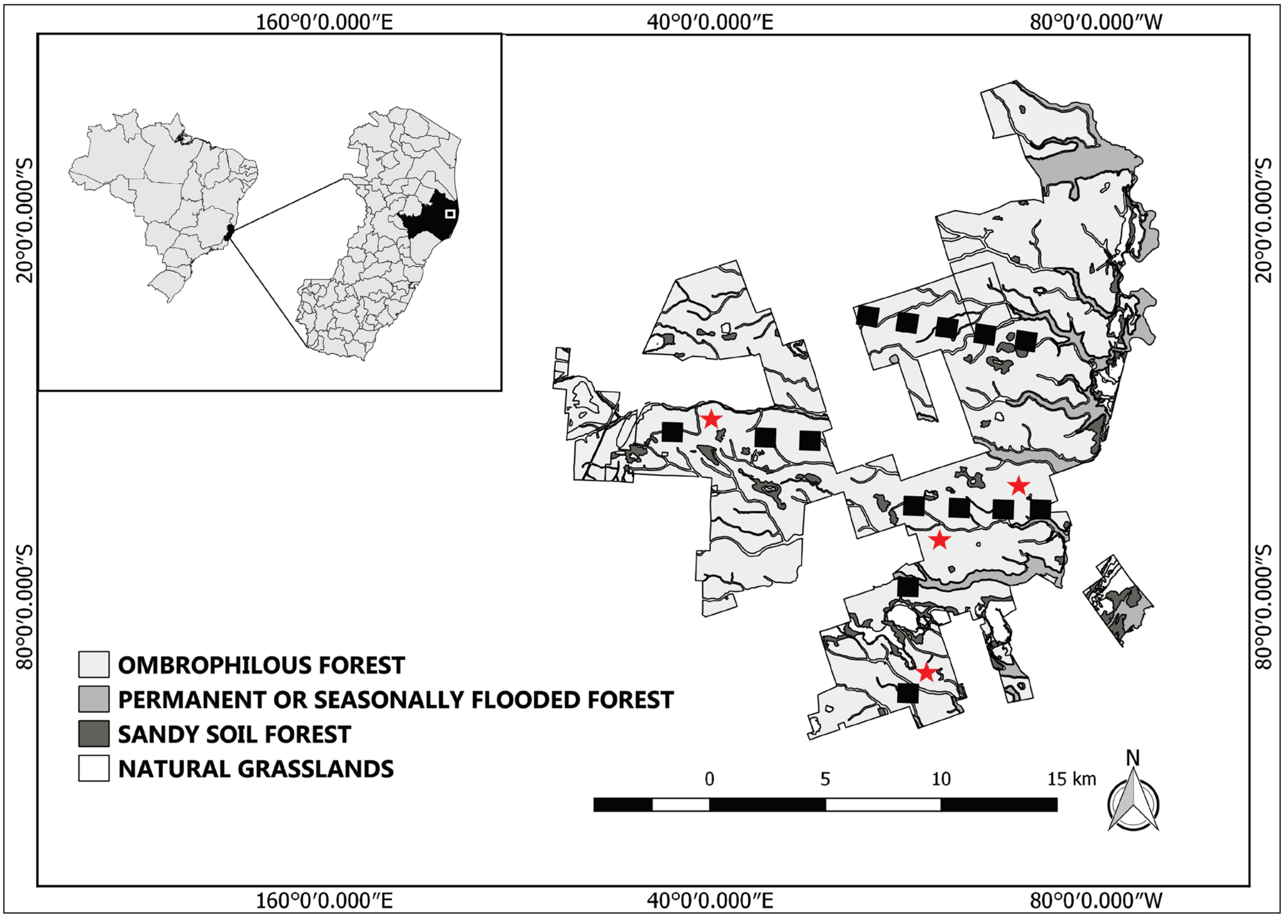


Figure 1. Location of the Vale Natural Reserve, north of Espírito Santo, southeastern Brazil, showing the vegetation types present in the reserve and the location of the plots (black squares) and collection sites of individuals for diet analysis and reproductive aspects (stars).

We estimated the frequency of the different microhabitats used by the treefrog in order to identify those preferentially used. We measured the distribution of heights that individuals were found to identify the range and preferred height that *A. brunoi* perched in the forest. We found some individuals occupying the hole of PVC tubes (used to demarcate the plots in the forest), but we did not consider such records for microhabitat usage estimates because they constituted artificial microhabitats.

We analyzed diet composition in 15 individuals collected outside areas of the plots, in order to avoid interference with our density estimates within plots. The frogs were euthanized with a topical anesthetic gel (lidocaine 5%), fixed in 10% formalin solution and preserved in 70% alcohol (IBAMA license 46327-4). The treefrog specimens were deposited in the Museu Nacional, Rio de Janeiro (MNRJ). We measured snout-vent length (SVL) and jaw width (JW) of the frogs using a Vernier Caliper (to the nearest 0.1 mm) and weighed them using a Pesola dynamometer (to the nearest 0.1 g). Individuals were dissected to determine the sex and to analyze stomach contents under a stereomicroscope. Animal prey items were measured (to the nearest 0.1 mm) using a Vernier Caliper and categorized to the taxonomic level of order (or family in the case of ants). Unidentified arthropod remains were grouped in a separate category "unidentified arthropod remains" (U.A.R). We measured the length (L) and width (W) of each prey item and its volume (V) was estimated using the ellipsoid formula: $V = 4/3\varpi (L/2) (W/2)^2$ (Dunham 1983). Diet composition was estimated in terms of number (N), volume (V, in mm^3) and frequency (F) of occurrence (percentage of stomachs containing a particular prey category) of each prey type in the stomachs. We estimated an index of relative importance (I_x) of each prey category in the diet which represents the sum of percentages of the number, volume and frequency of each prey type: [I_x = (%N +%V +%F)] (Powell et al. 1990). The effect of frog mouth width (in mm) on the volume (in mm^3) of the largest prey consumed was estimated by simple Regression Analysis.

For each gravid female, we recorded the mass of each ovary using an electronic scale (precision of 0.001 g), counted the total number of mature oocytes in both ovaries and measured the diameter of ten ovarian oocytes from each individual female using digital calipers (to the nearest 0.1 mm). We estimated female reproductive effort by dividing the total mass (g) of eggs by the total female body mass (g) including egg mass (Prado et al. 2000). We did not perform a regression analyses between female body size (SVL mm) and the respective number of oocytes due to the small sample size of gravid females (N = 4). We calculated an egg diameter effort index and a number of oocytes effort index by dividing the mean egg diameter and the mean number of oocytes, respectively, by the mean SVL. The results are represented as the mean ± SD and the range of data, the smallest and the largest number. Voucher specimens of *A. brunoi* are deposited in the Museu Nacional do Rio de Janeiro (MNRJ) under the voucher numbers Linhares, MNRJ 91008, 91009, 91010, 91011, 91012, 91013, 91014, 91015, 91016, 91017, 91018, 91019, 91020, 91021, 91022.

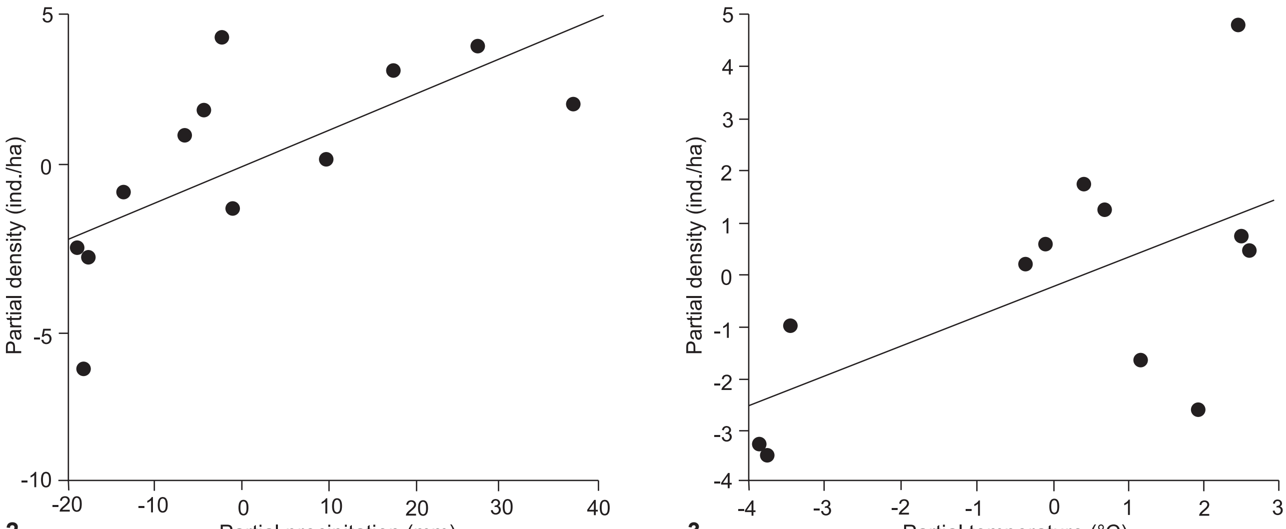
RESULTS

We recorded a total of 77 individuals of *A. brunoi*, all of them by visual encounters, in the ombrophilous forest. Treefrogs occurred in all months throughout the study, except July and September 2015, when no individuals were found. The abundance of individuals varied consistently throughout the year, with most individuals active during the rainy season (October to March) (n = 62; 80.6% of all individuals recorded) compared with that in the dry season (April to September) (N = 15; 19.4%). The estimated density of *A. brunoi* for the area varied markedly between the months of dry season (x = 1.79 + 1.90 ind/ha) and months of rainy season (x = 8.06 + 2.81 ind/ha) (t-test = 5.247, t = <0.001, n = 12). The relationship between the accumulated rainfall of the sampling period in each month (p = 0.01) and temperature (p = 0.04) was significantly related with corresponding density of individuals active in that particular month (Multiple Regression Analysis; $F_{2\text{-}9} = 10.328$; $R^2 = 0.695$; $p = 0.005$; n = 12; Density = -15.156+0.115*Rainfall+0.636*Temperature) (Figs 2, 3). In contrast, the accumulated humidity of the sampling period in each month was not significantly related to the corresponding density of active individuals in that particular month ($F1_{-10} = 0.083$; $R^2 = 0.008$; p = 0.77; n = 12).

In relation to daily activity, the first individuals were found active at dusk, from 06:00 pm, period at which we registered the highest number of individuals. Then, the number of active individuals decreased steadily till 22:00. Before 06:00 pm and after 10:00 pm no individuals of *A. brunoi* were found (Fig. 4).

In relation to use of the microhabitat, *A. brunoi* (n = 51) used seven different types of microhabitats in the forest, tree-trunks (51%; n = 26) and lianas (23.5%; n = 12) being the most frequent microhabitats used (Fig. 5, Suppl. material 1: Table S1). Individuals were found perched from 10 to 500 cm above the ground with a median perch height of 40 cm (first quartile = 20 cm, third quartile = 100 cm, n = 77).

We analyzed the stomach content of 15 *A. brunoi* individuals (8 males and 7 females) (x = 54,4 ± 6,8; 46.0–66.0 mm SVL; 5.5–46.0 g) and, of these, three stomachs were empty. *Aparasphenodon brunoi* consumed nine different types of prey in its diet (Table 1). The most representative were Orthoptera (20%) and Acari (16%). However, Phasmatodea dominated in volume



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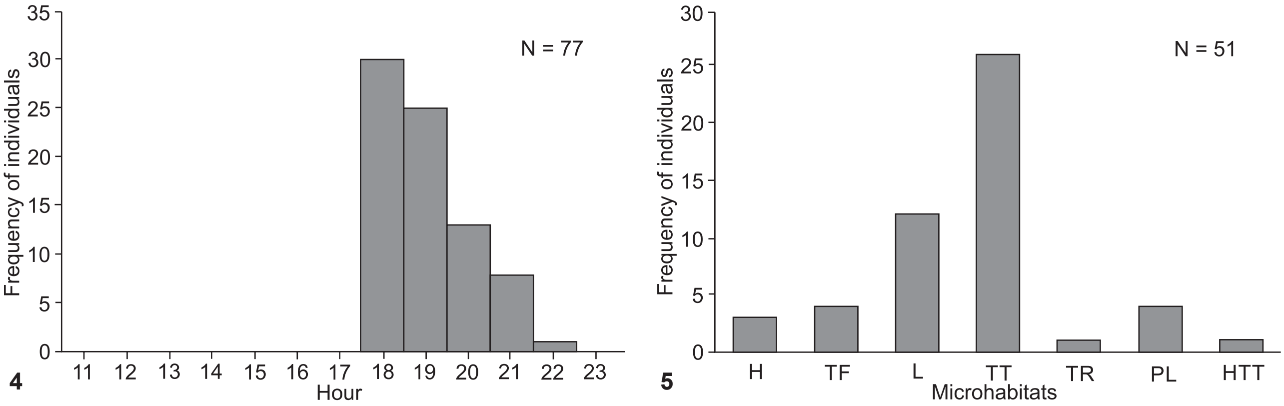
Figures 2–3. Results of Multiple Regression Analysis between (2) the accumulated rainfall of the sampling period in each month (June 2015 – July 2016) and (3) temperature with corresponding density of active individuals of *Aparasphenodon brunoi* in the Vale Natural Reserve, municipality of Linhares, Espírito Santo, Southeastern Brazil (Density = -15.156+0.115*Rainfall+0.636*Temperature).



Figures 4–5. Activity and microhabitat use of *Aparasphenodon brunoi*: (4) Number of individuals of *A. brunoi* (N = 77) recorded between 11:00 am and 11:00 pm in transects in the Vale Natural Reserve (VNR), municipality of Linhares, Espírito Santo, Southeastern Brazil). (5) Use of natural microhabitats by individuals of *A. brunoi* (N = 51) in the VNR. (H) On herbaceous plant, (TF) on a fallen tree trunk, (L) on liana, (TT) on a tree trunk, (TR) on a tree root, (PL) on a palm leaf, or in a (HTT) hollow in a tree trunk.

Table 1. Diet of *Aparasphenodon brunoi* at the Vale Natural Reserve, north of Espírito Santo, Brazil. Number (N), volume (V mm³), frequency (F) and Importance index (Ix) of prey categories. U.A.R = unidentified arthropod remains.

Gut Contents	N (%)	V (%)	F (%)	Ix
Arachnida				
Aranae	2 (8)	314.3 (5.48)	1 (6.67)	20.15
Acari	4 (16)	0.06 (0.001)	3 (20)	36.00
Insecta				
Orthoptera	5 (20)	1008.57 (31.51)	3 (20)	71,51
Phasmatodea	1 (4)	1822.21 (31.75)	1 (6.67)	42.42
Isoptera	1 (4)	0.30 (0.01)	1 (6.67)	10.68
Lepidoptera	1 (4)	1117.61 (19.47)	1 (6.67)	30.14
Hymenoptera (ants)	1 (4)	0.20 (0.004)	1 (6.67)	10.67
Coleoptera	1 (4)	157.72 (2.57)	1 (6.67)	13.24
Larvae	2 (8)	245.04 (4.27)	2 (13.33)	25.6
Plant Remains	7 (28)	53.12 (0.93)	7 (46.67)	75.6
U.A.R	–	220.293 (3.84)	–	–
Total	25 (100)	5739.42 (100)	21	–

(31.7%) followed by Orthoptera (31.5%), and Orthoptera (20%) and Acari were the most frequent, with the same percentage (20%) and Larvae represented 13.3% of frequency (Table 1). In relation to the index of relative importance (I_x), Orthoptera dominated (71.51%) followed by Phasmatodea (42.42%). The relationship between mean prey volume and treefrog JW was significantly related ($F_{1-13} = 8.388$; $R^2 = 0.392$; $p = 0.012$; $n = 15$).

The mean number of oocytes (± 1 SD) per female was 900.8 ± 358.1 (535–1338; n = 4), with 455.8 ± 218.4 oocytes in the left ovary (183–641) and 445 ± 182.5 oocytes in the right ovary (320–715; n = 4). The mean oocyte diameter was 1.36 ± 0.13 mm (1.22–1.52 mm; n = 40) and mean volume was 1.34 ± 0.38 mm³. The average total oocyte mass was 1.8 ± 0.7 g (0.8–2.4_ g; n = 4) (Suppl. material 2: Table S2). The average female reproductive effort was 7.0 ± 2.0% (4–9%, n = 4). The eggs diameter effort index was 2% and the number of oocytes effort index was 1.4 oocytes/mm.

DISCUSSION

Our data indicated that *A. brunoi* is an essentially nocturnal treefrog species as most anurans (Duellman and Trueb 1994), which is active in the first hours of the night. The species is active throughout the year but with a higher intensity from November to March, coinciding with the rainy season in the area. The density of individuals during the wet season was considerably higher (4.7 times higher) than that recorded during the dry season. In fact, the only months which we did not find active individuals (July and September) were months of the dry season in the area. *A. brunoi* activity (80.6%) occurred mostly during the wet season. Also, the amount of rainfall accumulated during sampling period interacted with temperature to explain the number of individuals of *A. brunoi* active throughout the year. All these data together reinforce that annual activity of *A. brunoi* predominates during the wet season in the ombrophilous forest. Although in the Restinga environment of Praia da Neves (Espírito Santo state) Teixeira et al. (2002) recorded some individuals of this species in some months of both dry and wet seasons. At the restinga of Barra de Maricá (Rio de Janeiro state) activity of this treefrog was restricted to months of the rainy season (Britto-Pereira et al. 1988). This tendency of increased activity during the rainy season in the restinga environment may result from the fact that this season provides more source of humidity to *A. brunoi*. The importance of humidity to favor activity in many frog species is well known and mostly results from their permeable skin (and associated risks of desiccation) and the need of humidity for reproduction (Duellman and Trueb 1994). This may be the reason that in restinga habitats, an environment characterized by low availability of free water and high temperatures (Silva et al. 2011), *A. brunoi* tend to live relatively restricted to bromeliads using the water stored inside the tank of these plants as source of moisture (Britto-Pereira et al. 1988, Teixeira et al. 2002). In restinga habitats, during diurnal period (the hottest one), individuals of *A. brunoi* remain inside the tank of bromeliads whereas nocturnally they remain frequently outside these plants, on leaves of bromeliads and some other plants and trunks (Sazima and Cardoso 1980, Schineider and Teixeira 2001, Mesquita et al. 2004, Sluys et al. 2004). Conversely, in the ombrophilous forest of the VNR, our data indicated that *A. brunoi* used seven different types of microhabitats where it perches preferentially about 1 m above ground, the preferred microhabitats for this species in the forest were tree trunks and lianas and coincidently, not bromeliads. We believe that the increased moisture and milder environmental temperatures of the ombrophilous forest favors a wider range of microhabitats and allows the frogs to remain active throughout the year. Interestingly, from the 77 *A. brunoi* individuals recorded during our study, not a single frog was associated with bromeliads, although these plants are frequent and abundant in the VNR, especially in the non-forest vegetation types (Siqueira et al. 2014, pers. obs. of authors). This is suggestive that the observed differences in the use of bromeliads as microhabitats among forested and restinga environments may be associated with the role of these plants as a source of moisture for these treefrogs in restinga habitats, a question that remains to be investigated.

Our data indicated that in the ombrophilous forest *A. brunoi* is an arthropod predator, with Orthoptera, Phasmatodea, and Lepidoptera being the most important prey items in its diet. In restinga areas, a diet composed by arthropods with consumption of Insecta, Arachnida and Myriapoda has aslo been reported (Teixeira et al. 2002, Mesquita et al. 2004, Sluys et al. 2004). The relatively wide array of prey types consumed indicates that *A. brunoi* is not a selective species and the consumption of relatively active prey can indicate ambush foraging behavior in this species. This can be advantageous to some species that have low foraging velocity and thus, a comparatively lower intensity than active foraging species (Strussman et al. 1984). The inclusion of some relatively large preys as Orthoptera, Phasmatodea and Lepidoptera in the diet can be suggestive of a specific energetic need by this treefrog. Probably the relative preference of arthropods of a relatively large size may be related to the energetic balance of this relatively large-bodied arboreal treefrog (costs and benefits of ingesting large prey). This idea is also supported by our data that shows that about 40% of the variation in prey volume (size) ingested was explained by frog mouth size, an indicative that as the treefrog increases in size tend to prey on larger prey (volumes), probably to keep a positive energy balance. Also, a positive relationship between frog mouth size and ingested prey size is expected for predators which do not chew their prey and are limited by gape size (Lima and Moreira 1993, Maia-Carneiro et al. 2013). The low amount of plant matter in the frog's diet probably corresponds to plant parts ingested during attempts to capture prey, as has been suggested also for the diet of many other frog species (e.g. Martins et al. 2010, Machado et al. 2016).

Reproductive data showed that ovigerous females of *A. brunoi* produce on average about 900 oocytes per reproductive event with a reproductive effort of about 7%. This relatively high number of oocytes produced by reproductive event may be related to the relatively large body size of this arboreal treefrog. The diameter of each egg was relatively small ($x = 1.36 \pm 0.13$ mm) when compared to the diameters of the eggs of other treefrogs, such as *Hypsiboas faber* (1.92 ± 0.15 mm), *Aplastodiscus eugenioi* (2.31 ± 0.22 mm) and *Phasmahyla gutatta* (2.40 ± 0.25 mm) (Hartmann et al. 2010), and may result from a trade-off between number and size of oocytes produced in which females produce large clutches with smaller eggs, a reproductive strategy for female *A. brunoi* in the ombrophilous forest (an interesting issue to compare to populations in restinga environments).

We conclude that *A. brunoi* is a nocturnal arboreal treefrog, active throughout the year but having increased activity during the wet season, resulting from the large amount of rain. In the forest, the treefrog tend to perch mainly on tree-trunks and lianas about 1 m above ground (instead of using predominantly bromeliads as in restingas), where it feeds preferably on relatively large bodied arthropod prey. The reproductive strategy for females of this comparatively large arboreal treefrog in the ombrophilous forest is to produce a large amount of relatively small-sized eggs. When living in the ombrophilous forest *A. brunoi* may change some features of its ecology (e.g. marked difference in the use of bromeliads) compared to when living in restinga habitats. Our study is the first to gather information on the ecology of this treefrog in ombrophilous forest of the Atlantic Rainforest Biome of Brazil.

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Supplementary material 1

Table S1. Summary of observations of *Aparasphenodon brunoi* in Vale Natural Reserve, municipality of Linhares, state of Espírito Santo, Southeastern Brazil.

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Data type: (measurement/occurence/multimedia/etc.)

Link: https://doi.org/10.3897/subtbiol.34.20477.suppl1

Supplementary material 2

Table S2. Data of *Aparasphenodon brunoi* individuals collected outside plot areas for diet and reproductive aspects analysis.

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