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Article

Are ants attracted to herbivorized leaves of *Caryocar brasiliense* Camb. (Caryocaraceae)?

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Abstract: Different profiles of secondary compounds are released by plants after herbivore attack. Many of these compounds are used by predators and parasitoids to locate herbivores that are damaging leaves. Such an induced indirect defense was tested with the *Caryocar brasiliense*-ant system in the dry season, when *C. brasiliense* has old leaves, and in the rainy season, when *C. brasiliense* has new leaves. A total of 20 plants were analyzed per season. Two opposite leaves of the same branch were selected for each plant. Approximately 40% of the area of one leaf was removed (treatment leaf) while the other leaf remained intact (control). The number of ants that visited each leaf was counted simultaneously for a period of 15 minutes and the mean difference in ant number was tested by paired t-test. The mean number of ants differed significantly between treatment and control only in the rainy season (t = 3.004, df = 19, p = 0.007). This finding suggests the presence of induced defense in this system only when the leaves are young. The study supports the Optimal Defense Theory since young leaves of *C. brasiliense* with artificial damage attracted significantly more ants than leaves without damage and represents the first evidence of an induced defense mechanism in the *C. brasiliense*-ant system.

Keywords: *ant-plant interaction; artificial herbivory; Cerrado; induced defense; Optimal Defense Theory.*

As formigas são atraídas por folhas herbivoradas de *Caryocar brasiliense* Camb. (Caryocaraceae)?

Resumo: Diferentes compostos químicos são liberados pelas plantas após o ataque dos herbívoros. Muitos desses compostos são usados por predadores e parasitoides para localizar os herbívoros que estão injuriando as plantas. Esse tipo de defesa induzida indireta foi testada no sistema *Caryocar brasiliense* e formigas em duas estações: seca (quando *C. brasiliense* está com folhas maduras) e chuvosa (quando *C. brasiliense* está com folhas jovens). Nós analisamos 20 plantas por estação do ano. Em cada planta nós selecionamos duas folhas opostas de um mesmo ramo. Nós removemos cerca de 40% da área de uma das folhas, deixando a outra folha intacta. Nós mensuramos simultaneamente o número de formigas que visitaram cada tipo de folha por 15 min e analisamos a diferença entre o número de formigas em cada tipo de folha através de teste t pareado. Nós observamos diferença significativa no número de formigas que patrulham as folhas tratamento (com herbivoria artificial) e controle (sem hervivoria artificial) apenas na estação chuvosa (t = 3,004, df = 19, p = 0,007). Isso sugere que existe defesa induzida nesse sistema somente quando as folhas são jovens. Nosso estudo corrobora a Teoria de Defesa Ótima já que apenas as folhas jovens de *C. brasiliense* com dano artificial atraíram significativamente mais formigas do que as folhas sem danos. Esta é a primeira vez que mecanismos de defesa induzida são observados no sistema *C. brasiliense-formigas*.

Palavras-chave: *Cerrado; defesa induzida; herbivoria artificial; interação inseto-planta; Teoria da Defesa Ótima.*

Introduction

Plant defense strategies against herbivores can be categorized as either direct or indirect. Direct defenses negatively affect herbivore metabolism via toxic compounds and digestibility reducers (Speight et al. 1999, Marquis 2012) whereas indirect defenses occur when the plant attracts other insects that can reduce herbivore damage (Bixenmann et al. 2013). In the latter strategy, the plant provides chemical cues or rewards (food and/or shelter) to attract predators and parasitoids of the herbivores (Boege & Marquis 2005).

Leaves naturally release volatile organic compounds (VOCs) during their development. However, when damage occurs, usually due to herbivores, some plants quantitatively and/or qualitatively change their VOC profile (Paré & Tumlinson 1999, Marquis 2012). Such new VOC profiles may inhibit oviposition or reduce herbivore performance (induced direct defense), or act as a cue for predators and parasitoids to locate herbivores that are causing damage (induced indirect defense) (Turlings et al. 1995, Paré & Tumlinson 1999, Carroll et al. 2006, Heil 2014). Kost & Heil (2006) demonstrated that VOCs released by damage plants could be recognized by undamaged neighboring co-specific plants, acting as a trigger for increased secretion of extrafloral nectar.

Ants are known to respond to VOCs of host plants (Brouat et al. 2000), which generally attract ants to damaged leaves (Schatz et al. 2009). Three VOCs - methyl salicylate, 2E hexen-1-ol and hexanal - were detected in damaged leaves of *Leonardoxa africana* (Fabaceae) (Schatz et al. 2009), as well as in *Macaranga* (Euphorbiaceae) (Inui & Itioka 2007). Most studies that have tested the effectiveness of chemical signals for attracting ants after plant damage used myrmecophytic systems, such as *Hirtella myrmecophila* (Chrysobalanaceae)-*Allomerus octoarticulatus* ants (Romero & Izzo 2004), *Tachigali myrmecophila* (Fabaceae)-*Pseudomyrmex concolor* ants (Pacheco & Del-Claro 2018), *Piper* (Piperaceae)-*Pheidole* ants (Mayer et al. 2008), and *Cecropia* (Urticaceae)-*Azteca* ants (Agrawal 1998). Analysis of VOCs after damage to myrmecophytic and non-myrmecophytic plants showed that the chemical profiles differed with only the myrmecophytic species (Mayer et al. 2008). Unfortunately, studies of VOCs of non-myrmecophytic plants are scarce.

Caryocar brasiliense Camb. (Caryocaraceae), known locally as "pequizeiro", is a typical tree of the Brazilian Cerrado. It is a semi-deciduous plant that loses some of its leaves during the dry season (May to July) (Araujo 1995), and grows young leaves during the beginning of the rainy season (September to March) (Vilela et al. 2008). Flowering starts soon after the expansion of young leaves, with fruiting occurring from October to February (Vilela et al. 2008).

According to Oliveira & Freitas (2004) and Oliveira et al. (2012), the main herbivores of *C. brasiliense* are: (1) *Eunica bechina* (Lepidoptera: Nymphalidae), whose larvae feed on young leaves; (2) *Edesa rufomarginata* (Hemiptera: Pentatomidae), which feeds on plant sap; (3) *Prodiplosis floricola* (Diptera: Cecidomyiidae), which the larvae feed on flower buds; and (4) wasps (Hymenoptera: Chalcidoidea), whose larvae induce gall formation in leaves and branches. Plants of *C. brasiliense* are constantly visited (day and night) by more than 30 species of ants, especially of the genera *Camponotus* and *Cephalotes*, which feed on secretions of extrafloral nectaries (EFNs) of flower buds and young leaves (Oliveira & Brandão 1991).

Although several studies have verified the importance of ants as a constitutive defense in *C. brasiliense* (e.g., Oliveira 1997, Oliveira & Freitas 2004), the existence of an induced indirect defense in this system has not been tested. This study aimed to determine if this type of defense occurs in the *C. brasiliense*-ant system. Specifically, the following questions were addressed: (1) do *C. brasiliense* leaves with artificial damage attract more ants than leaves without damage? (2) Are young leaves more attractive to ants than mature leaves?

Material and Methods

Experiments were conducted in Emas National Park (ENP) located in the state of Goiás, Brazil (17°49'-18°28'S; 52°39'-53°10'W) during the dry (July 2012) and rainy (September 2012) seasons with 20 *Caryocar brasiliense* plants per season. The plants had mature leaves and considerable herbivory during the dry season (Figure 1a), while EFNs were active and young leaves did not have signs of herbivory during the rainy season (Figure 1b).

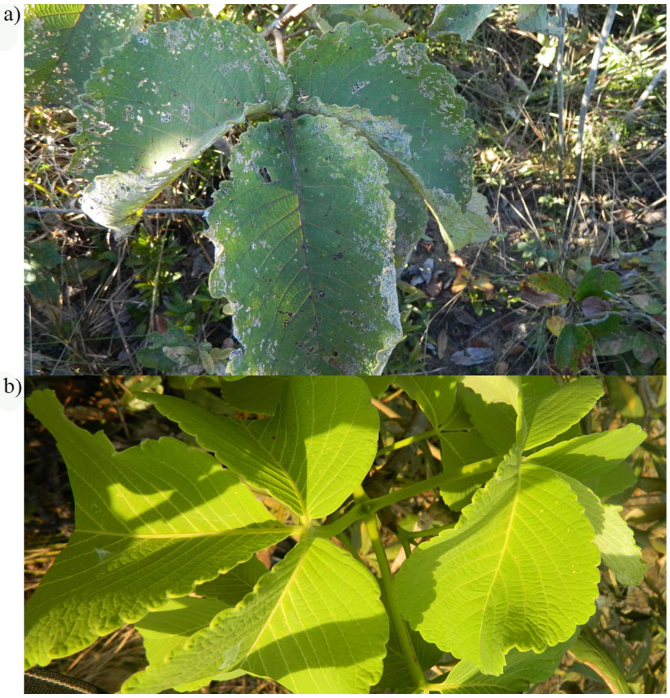

Figure 1. Leaves of *Caryocar brasiliense* in the dry (a) and in the rainy (b) seasons.

A total of 40 *C. brasiliense* trees of similar height, architecture and herbivory level were selected for study. One branch with fully expanded leaves and minimal herbivore damage was selected in each plant, on which two opposite leaves were identified. Approximately 40% of the leaf area of one leaf was removed with a scissors (treatment leaf, with artificial herbivory), while the other leaf remained intact (control leaf, without artificial herbivory). Both leaves were shaken prior to the start of the experiment to assure no ants were present.

The number of ants visiting paired leaves was counted simultaneously for period of 15 minutes. Ants that were observed visiting the experiment leaves were manually collected with a forceps, preserved in 70% alcohol, and identified to morphospecies. Difference in mean number of ants was tested by paired t-test, considering plants as sample units.

Results

Nine morphospecies of five subfamilies of ants occurred on the leaves of *C. brasiliense* during our study. Three of the morphospecies occurred only in the dry season, two only in the rainy season and four in both seasons. Different morphospecies of ants patrolled the experimental plants, but in most cases only one morphospecies was found on a plant. *Camponotus* was the most diverse genus with three morphospecies (Table 1).

Table 1. Ant species (Hymenoptera: Formicidae) that patrolled plants of *Caryocar brasiliense* in Emas National Park in the dry season (July) and in the rainy season (September) of 2012.

Taxon		Season	
Subfamily	**Morphospecies**	**Dry**	**Rainy**
Dolichoderinae	*Azteca* sp. 1	X	X
	Azteca sp. 2	X	X
Formicinae	*Camponotus* sp. 1	X	X
	Camponotus sp. 2	X	X
	Camponotus sp. 3	X	
Myrmicinae	*Cephalotes* sp. 1	X	
	Crematogaster sp. 1	X	
Ponerinae	*Pachycondyla* sp. 1		X
Pseudomyrmecinae	*Pseudomyrmex* sp. 1		X

There was no significant difference in the mean number of ants between treatment and control leaves in the dry season (t = 1.011, df = 19, p = 0.325; mean = 5.75 and 4.20, respectively) (Figure 2a), but there was significantly more ants on treatment (mean = 4.15) than control (mean = 2.10) leaves in the rainy season (t = 3.004, df = 19, p = 0.007) (Figure 2b).

Discussion

Although the mean number of ants on treatment leaves was greater than that of control leaves in both seasons, the difference was only significant in the rainy season, suggesting induced defense in this system only when leaves are young.

Herbivory of young leaves tends to have a greater effect on plant fitness than does herbivory on mature leaves (Jurik & Chabot 1986). In addition, young leaves have not yet contributed enough in terms of photosynthetic production to offset the high costs of their construction (Radhika et al. 2008). Because young leaves are softer and more nutritious than mature leaves they tend to be more attractive to herbivores (Kursar & Coley 2003). For example, Coley & Barone (1996) found that 70% of leaf herbivory of rainforest plants occurs during leaf expansion.

The Optimal Defense Theory (ODT) predicts that resource allocation for defense should be higher in the most valuable plant parts (measured in terms of reduction in fitness by its removal) and parts more likely to be damaged by herbivores (Mckey 1974, Zangerl & Bazzaz 1992). Many studies have found young leaves to have higher concentrations and a greater diversity of secondary compounds than mature leaves (e.g., van Dam et al. 1995, Read et al. 2003), which supports ODT. Radhika et al. (2008), for example, applied jasmonic acid to castor bean leaves and found that the release of volatiles and extrafloral nectary secretion was higher in young than in old leaves. The present study also supports ODT, since only the young leaves of *C. brasiliense* with artificial herbivory attracted significantly more ants than leaves without artificial herbivory. Likewise, Pacheco & Del-Claro (2018) also detected a higher number of *Pseudomyrmex concolor* ants on young damaged leaves of *Tachigali myrmecophila* in the Amazon.

Although the present study did not analyze the composition and concentration of leaf compounds, the response of ants (higher recruitment in damaged young leaves) indicates chemical signaling by the plant after damage, suggesting that induced defense is only evident when leaves are young. The present results may be an underestimation because of the type of artificial herbivory employed (damage by cutting with scissors), because some studies have shown that natural herbivory elicits greater defense induction than mechanical damage (Hartley & Lawton 1987, Massey, Ennos & Hartley 2007, Quigley & Anderson 2014). Oral secretions provide herbivore-specific cues for defense induction by many insects (Alborn et al. 1997, Tian et al. 2012). Components of insect saliva, plant cell wall fragments and other cues create a signal cascade that triggers a defense response that increases concentrations of secondary metabolites (Stam et al. 2014). While counting ants in the present study, many were observed touching the region where the leaves had been cut with their antennae and/or mandibles, suggesting that liquid substances, in addition to volatile compounds, may also be acting as signals for the ants.

Plants of *C. brasiliense* are constantly visited by ants that feed on extrafloral secretions of flower buds and young leaves (Oliveira & Brandão 1991). Several studies have shown that ants reduce herbivore damage to *C. brasiliense* (e.g., Oliveira & Freitas 2004), thereby increasing plant fitness (Oliveira 1997). The observations of the present study suggest that there is induced defense in young leaves of *C. brasiliense* and represent the first evidence of an induced defense mechanism in the *C. brasiliense*-ant system. Additional studies, including quantitative and qualitative chemical analyses, are recommended to further investigate the role of this type of defense in *C. brasiliense*. Additional research on VOCs in other non-myrmecophytic systems are needed. Such studies could lead to greater insight into whether particular chemical traits are associated with the evolution of ant-plant relationships.

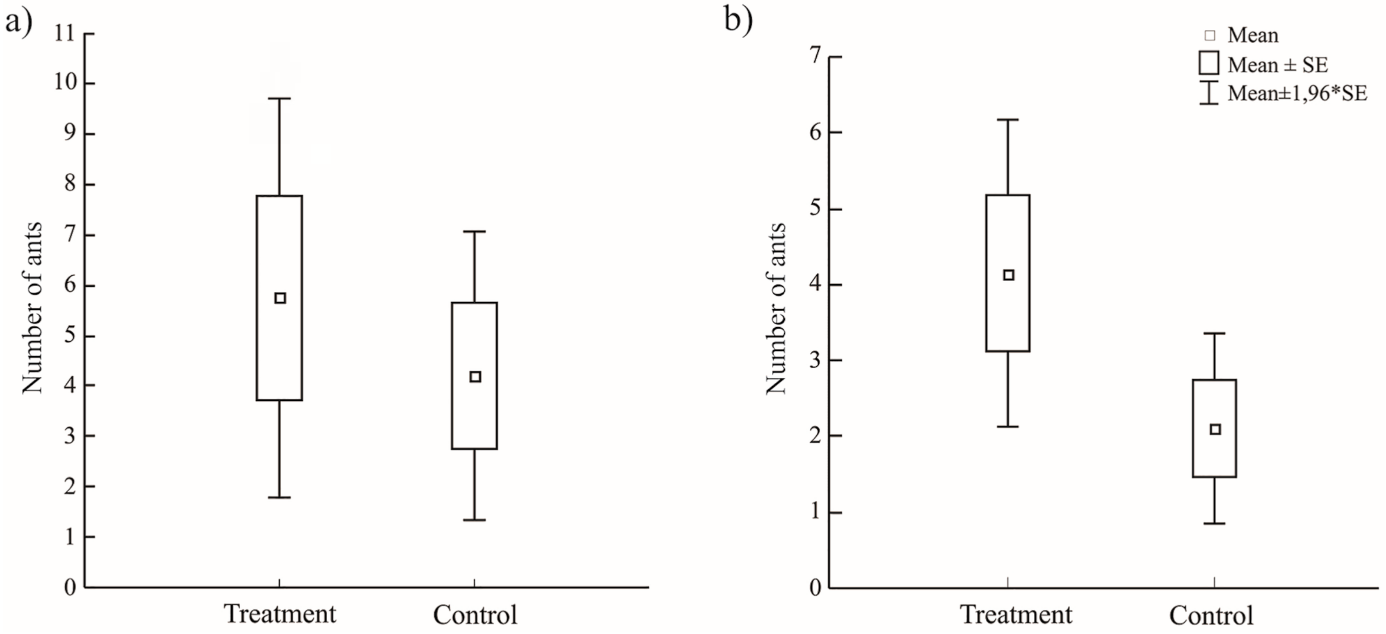

Figure 2. Mean number of ants that visited treatment (with artificial herbivory) and control (without artificial herbivory) leaves of *Caryocar brasiliense* in (a) the dry season (t = 1.011, df = 19, p = 0.325) and (b) in the rainy season (t = 3.004; df = 19; p = 0.007). Twenty plants were sampled in each season.

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Authors Contributions

Verônica Bernardino de S. Magalhães: Contributed with data acquisition, data analyses and manuscript preparation.

Viviane Gianluppi Ferro: Contributed with data acquisition, data analyses and manuscript preparation.

Conflict of Interest

The authors declare that they have no conflict of interest related to the publication of this manuscript.

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Bats and COVID-19: villains or victims?

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Abstract: Since the beginning of the COVID-19 pandemic, bats are being pointed as responsible for its origin, even without solid scientific evidence. In this opinion piece, we discuss the most updated information on bats and COVID-19 and argue that bats should not be blamed for a disease they are not responsible for. Bats should be seen not as dangerous animals but, instead, as sources of several scientific insights useful for human health.

Keywords: Chiroptera; spillovers; wildlife; zoonoses; zoonotic spillovers.

Morcegos e a COVID-19: vilões ou vítimas?

Resumo: Morcegos e COVID-19: vilões ou vítimas? Desde o início da epidemia de COVID-19, morcegos estão sendo apontados como os culpados por sua origem, mesmo sem evidências científicas sólidas que apontem para tal. Neste artigo de opinião trazemos informações atualizadas sobre morcegos e COVID-19 e discutimos porque morcegos não deveriam ser culpados por uma doença pela qual não foram responsáveis. Morcegos não deveriam ser vistos como animais perigosos, mas sim como fontes de várias ideias e abordagens científicas úteis para a saúde humana.

Palavras-chave: Chiroptera; doenças zoonóticas; transferência de patógenos; vida silvestre; zoonoses.

Since the beginning of the COVID-19 pandemic, we have been flooded by information pointing to bats as responsible for its origin. However, there is no consensual, categorical study to date showing a direct connection between bats and the SARS-CoV-2 virus, responsible for the COVID-19 pandemic. On the contrary, the available studies point to the existence of an intermediate host, whose identity is unknown at the moment. The search for this host began with the sequencing of the SARS-CoV-2 genome (Wu et al. 2020), the virus causing the disease in humans. The genome of a coronavirus (RaTG13) previously isolated from *Rhinolophus affinis* bats in China is about 96% similar to the SARS-CoV-2 genome (Zhou et al. 2020). Although the RaTG13 coronavirus has high genetic similarity to SARS-CoV-2, there are divergences in the viral receptor ligand domain (RBD) region, suggesting that this bat coronavirus cannot efficiently bind to human ECA2. On the other hand, a pangolin coronavirus has a strong resemblance to SARS-CoV-2 in the RBD region, which supports the hypothesis of an intermediate host.

This genetic distance suggests that SARS-CoV-2 and RaTG13 diverged about 40 to 70 years. In other words, decades have passed since these viruses shared a common ancestor, but since then those viruses differentiated (Zhou et al. 2020). Such divergence may also point to the existence of not just one, but multiple intermediate hosts. Pangolins (*Manis javanica*) have been identified as this potential intermediate host, a hypothesis that has not yet been ruled out. However, coronaviruses isolated from pangolins also proved to be too distinct to represent the direct ancestors of SARS-CoV-2 (Lam et al. *in press*). Snakes had also been suggested as potential intermediate hosts, a possibility that also seems to have been refuted. There is consensus among scientists that SARS-CoV-2 originated from wildlife (Fam et al. 2020) but, in fact, this origin has not yet been found in nature.

Bats have received considerable attention in relation to coronaviruses, mainly because they carry a great variety of such viruses and are their natural and ancestral reservoirs (Ge et al. 2015). Moreover, since the SARS epidemic, in 2002, their mobility – acquired through flight – was identified as a characteristic that could enhance pathogen dispersion. In consequence of this attention, more viruses were found in this group. As would be expected, rodents and bats, the most diverse groups among mammals harbor viruses proportional to their diversity, but a recent study clearly shows that bats and rodents - usually the ‘villains’ pointed out in epidemics - are neither more nor less important as reservoirs of viruses with potential for contamination in humans than other animals. In fact, there is a relationship between greater potential for virus transmission among mammals with high abundance, such as those we raise and with whom we maintain greater contact, such as goats, pigs and cattle (Mollentze & Steicker 2020).

Our understanding of the complex viral transmission dynamics from wildlife to humans will remain limited until we have carefully searched for viruses circulating in a much more representative sample of all animal taxa.

Fam et al. (2020) suggest that SARS-CoV-2 has become a human specialist. This means high performance to infect *Homo sapiens* and less ability to have the same efficiency to infect other species unless new mutations and/or recombination create a derived coronavirus capable of breaking the species barriers again, fueling this never-ending arms race between hosts and pathogens. Such a scenario demonstrates the complexity of the process, and the complexity of the topic itself for non-specialists. Furthermore, unfortunately, the details of the intricate and intriguing network of relationships between pathogens, primary and intermediate hosts, their transmission mechanisms, and the possibilities of associated mutations are not always conveyed in a complete and correct manner to the general public, contributing to misguided and hasty interpretations. In doubt or in a hurry to point out a 'culprit' for COVID-19, bats ended up being held responsible for something they were not directly involved in. And the results have been extremely negative for a group that, unfortunately, already suffers from an unfair negative reputation among the public opinion.

Reports of increased cases of persecution, vandalism, and destruction of roosts and bat populations have boomed around the world since the beginning of the pandemic (Fenton et al. 2020, Zhao 2020). Such acts will negatively affect the conservation of the group and the irreplaceable ecosystem services provided by the 1421 species of bats described to date (Wilson & Mittermeier 2019). Such services include the pollination of hundreds of plant species, many of which are of economic interest, seed dispersal and habitat regeneration, the control of insect populations, including agricultural 'pests' and disease vectors for humans and commercial animals (Kunz et al. 2011; Ghanem & Voigt 2012).

Condemning bats is also a mistake from a scientific point of view. Several studies have shown that bats have high resistance to viral infections, not reacting with the typical inflammatory response that occurs in several animals, including humans (Hayman 2019; Huang et al. 2019). This inflammatory response is a way of fighting infection, but when it exceeds certain levels, it may cause pathological damage to organisms, contributing to aging and age-related diseases. Bats have a natural ability to mitigate inflammation caused by stress and infection, therefore being excellent models to understand how to deal with viral infections (Huang et al. 2019). Mechanisms of gene expression and self-repair in bat DNA – not seen in humans or other mammals – also seem to explain another unique characteristic of this group: its longevity, which is much higher than that expected for animals of their size (Teeling et al. 2018). This makes bats ideal models for finding solutions to slow the aging process, as well as finding treatments for age-related diseases in humans. Further, bats have low susceptibility to cancer (Huang et al. 2019), and the oral microflora of some species, for example, appears to decrease susceptibility to caries (Brändel et al. 2013). These should be reasons enough to look at these animals as potential reservoirs, not of diseases, but of extremely useful information for humans. But in the midst of a pandemic – and in the search for a 'culprit' – this importance seems irrelevant.

Dangerous are also the alarmist views pointing to the enormous risk we would be taking because we live with many species and, consequently, with the viruses, bacteria and other microorganisms that they host (e.g. Lapola 2020). Should we blame our rich biodiversity for spillovers caused not by the animals themselves, but by human action and the way we interact with the natural environment? Obviously not. All animals, including mammals – and we humans are no exception – are natural hosts for thousands of species of microorganisms, but only a small portion of this biota has lethal potential, and those with pandemic lethal potential are even fewer. Brazil is a megadiverse country, third in the world in terms of bat species richness (182 so far – Nogueira et al. 2018), first in the world in number of primate species, being among the top three of birds, amphibians and reptiles, among several other animal and plant groups. Attributing a direct causal relationship such as 'more species, more viruses, more danger' is not correct, simply because such a relationship does not necessarily exist. There are several other variables that need to be considered in a zoonotic contamination process.

Zoonotic spillovers are complex processes (Plowright et al. 2017; Ellwanger & Chies 2018), but the knowledge accumulated so far shows that their occurrence may not result from the direct presence of animals, and even less from high levels of biodiversity, but from human action and how we interact with the natural environment. Indeed, the reduction in the number of species and their habitats seems to be a much more direct cause for the spread of diseases of wildlife origin (Ezenwa et al. 2006; Keesing et al. 2010). In fact, several studies point to the existence of the so-called 'dilution effect', i.e., in the presence of several potential host species of the same virus (or its variants), the encounter rate between infected individuals and individuals likely to be infected decreases, transmission rates decrease, as well as decreases the density of infected individuals in populations of the different species present (Ostfeld & Keesing 2000; Schmidt & Ostfeld 2001; Swaddle & Calos 2008). Interestingly, it was recently proposed that the reduction in the dispersion of pathogens that followed the extinction of the mammalian megafauna at the end of the Quaternary might have led to an increase in the emergence of diseases of zoonotic origin in humans (Doughty et al. *in press*). The fact is that the development model that we insist on following is the cause of the environmental imbalances that we are witnessing, is at the origin of the COVID-19 pandemic, and will be the cause of others that will follow if we do not change our action on the planet. Simplistically associating bats, or any other animals, with zoonotic spillovers is clearly a disservice both to them and to humans. Service will be to take advantage of this moment to become aware that our depredatory actions on habitats and species also have consequences for our survival as individuals, perhaps as a species, and with that beginning to change the way we act on our planet. There is no lack of papers showing the link between environmental imbalances and emerging infectious diseases in the literature (e.g. Daszak et al. 2001, Weiss & McMichael 2004, Jones et al. 2008 Ellwanger et al. 2020). However, there is a lack of effective actions to avoid the environmental imbalances that are consequences of the predatory advance on natural areas by human activities such as urbanization (Rezende et al. 2018, Favoretto et al. 2019), agriculture (Fernandes et al. 2013, Andrade et al. 2016) and mining (Rotureau et al. 2006; Tuesdays-Trettel et al. 2019).

As we wrote this point of view, an article by Thomas Lovejoy, a renowned conservationist with several studies in the Neotropical region, was published in the National Geographic magazine (May 2020). We took the liberty of concluding by reproducing an excerpt of his words in that article: *"Nature sustains us. It's where we originated. The lesson for humanity from this pandemic is not to be afraid of nature, but rather to restore it, embrace it, and understand how to live with and benefit from it. All that biodiversity is essentially a gigantic library of solutions, pretested by natural selection and evolution, to various biological challenges. The idiosyncratic biology of bats, for example—the fact that they are somehow immune to the coronavirus—might contribute to development of a treatment in humans. Humanity has huge respect for libraries of our own works; there is every reason to treat the living library of nature with the same respect and care."*

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Author Contributions

Maria João Ramos Pereira: Contributed equally to manuscript preparation.

Enrico Bernard: Contributed equally to manuscript preparation.

Ludmilla M. S. Aguiar: Contributed equally to manuscript preparation.

Conflict of interest

MJRP, EB and LMSA are, respectively, Vice-President, President and Founder and Coordinator of the Cerrado Secretariate of the Sociedade Brasileira para o Estudo de Quirópteros – SBEQ (Brazilian Bat Research Society).

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Inventory

Knowing biodiversity: Fishes from the Guareí River basin, a tributary of the Jurumirim reservoir, Paranapanema River, Brazil

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Abstract: The Guareí River is a tributary of the Paranapanema River (Brazil), located in the upper portion of the Jurumirim Reservoir. Fish fauna studies in this watershed began in the 2000s, but they were restricted to a few waterbodies. This work conducted a broad survey of the fish fauna in tributary streams and the main channel of the Guareí River. Sampling occurred between February 2017 and November 2018 at 36 sites and using different collection methods. We captured 2,169 specimens belonging to 50 species, 16 families and 6 orders. The species accumulation curve tended to stabilize but indicated that species richness is underestimated. Almost all species are native (46); only three were non-native (*Hyphessobrycon eques, Oreochromis niloticus* and *Poecilia reticulata*) and one was undefined (*Gymnotus pantanal*). Among the native species, two are unknown to science (*Bryconamericus* aff. *iheringii* and *Hypostomus* sp. n.) and three are migratory (*Leporinus friderici, Megaleporinus obtusidens* and *Pimelodus maculatus*). In this paper, we provide images of species collected. Results indicate that the Guareí River basin is a hotspot of fish diversity in the Upper Paranapanema River, stressing the need for adequate management and conservation actions.

Keywords: *Inventory; migratory fish; non-native fish; Paraná River basin; stream; undescribed species.*

Conhecendo a biodiversidade: Peixes da bacia do Rio Guareí, um afluente do reservatório de Jurumirim, Rio Paranapanema, Brasil

Resumo: O Rio Guareí é um afluente do Rio Paranapanema (Brasil), localizado na parte superior do reservatório de Jurumirim. Os estudos da fauna de peixes nesta bacia hidrográfica começaram nos anos 2000, mas estão restritos a poucos corpos d'água. Este trabalho realizou um amplo levantamento da ictiofauna em tributários e no canal principal do Rio Guareí. As amostragens ocorreram entre Fevereiro de 2017 e Novembro de 2018 em 36 sítios e utilizando diferentes métodos de coleta. Capturamos 2.169 espécimes pertencentes a 50 espécies, 16 famílias e 6 ordens. A curva de acumulação de espécies tendeu a se estabilizar, mas indicou que a riqueza de espécies está subestimada. Quase todas as espécies são nativas (46); apenas três não nativas (*Hyphessobrycon eques*, *Oreochromis niloticus*, e *Poecilia reticulata*) e uma não definida (*Gymnotus pantanal*). Entre as espécies nativas, duas são desconhecidas da ciência (*Bryconamericus* aff. *iheringii* e *Hypostomus* sp. n.) e três migradoras (*Leporinus friderici*, *Megaleporinus obtusidens* e *Pimelodus maculatus*). Neste artigo, fornecemos imagens das espécies coletadas. Os resultados indicam que a bacia do Rio Guareí é uma região importante em termos de diversidade de peixes no alto Rio Paranapanema, enfatizando a necessidade de ações adequadas de manejo e conservação.

Palavras-chave: *Bacia do Rio Paraná; espécies não descritas; inventário; peixe migrador; peixes não nativos; riacho.*

Introduction

The diversity of freshwater fishes in Brazil (> 3,000 species; ICMBio 2018) is higher than in any other Neotropical country (e.g., Litz & Koerber 2014; Mirande & Koerber 2015; DoNascimiento et al. 2017). In part, the high diversity is related to the presence of the Amazon basin, a megadiverse region (Dagosta & de Pinna 2019). However, other important river systems hold remarkable biodiversity, such as the Upper Paraná River (Langeani et al. 2007; Ota et al. 2018). This basin sums more than three hundred species, with dozens still undescribed (Langeani et al. 2007).

In the last century, numerous studies about fish composition, biology, ecology, taxonomy have been carried out in the Upper Paraná River (e.g., Agostinho & Júlio-Jr. 1999; Britto & Carvalho 2006; Brandão et al. 2009; Dias & Tejerina-Garro 2010; Esguícero & Arcifa 2011; Cionek et al. 2012; Carvalho & Langeani 2013; Santos et al. 2017; Frota et al. 2020), and this basin is possibly the most studied in Brazil. However, several questions remain open, including total diversity, biogeographic patterns and ecological aspects, demanding further studies. The occurrence of *Phenacorhamdia tenebrosa* (Schubart, 1964) on the Upper Paraná River is a good example: although this fish was recorded by several studies (e.g., Schubart 1964; Manoel & Uieda 2018; Cavalli et al. 2018; Vicentin et al. 2019), more detailed information on its distribution is lacking. Perhaps more important is the fact that several waterbodies remain uninvestigated, especially small and mid-size water courses, which may harbor species unknown (i.e., undescribed) to science (Langeani et al. 2007).

If the Upper Paraná River is the most studied, it is also the most disturbed (Agostinho et al. 2007). Urbanization, agriculture, invasive species and hydrological alterations related to human activities have caused significant perturbations to fish diversity. The construction of hydropower dams, in particular, has played a central role inducing habitat alterations and biodiversity losses (Agostinho et al. 2008; Pelicice et al. 2017). For instance, the main tributaries of the basin, such as the Tietê, Grande and Paranapanema rivers, are regulated by cascade of dams (Petesse & Petrere Jr. 2012; Loures & Pompeu 2018; Pelicice et al. 2018), blocking migratory dynamics and affecting population recruitment (Pelicice & Agostinho 2008). Ichthyological surveys remain incomplete in these highly disturbed systems, and once conservation initiatives rely mainly on those data (Azevedo-Santos et al. 2019), more research is needed to provide basic information in these impacted waterbodies.

Tributaries play important roles in impounded areas (Nunes et al. 2015; Silva et al. 2015; Marques et al. 2018), since they preserve fluvial conditions and a natural or semi-natural flow regime, reducing the impacts on the environment and biodiversity. The presence of tributaries may explain the maintenance of fish diversity in impounded areas, because reservoirs impose serious constraints to fish recruitment (Agostinho et al. 2008). Some studies in the Paranapanema River have pointed to the importance of tributaries (e.g., Hoffmann et al. 2005; Pelicice & Agostinho 2008). The Jurumirim Reservoir, in particular, has been investigated by multiple studies concerning its aquatic fauna (e.g., Panarelli et al. 2001; Sartori et al. 2009), including fish (e.g., Castro et al. 2003a; Lima et al. 2016; Lima et al. 2018; Queiroz-Sousa et al. 2019). Some studies (e.g., Seabra et al. 2012; Nobile et al. 2019) provided information about fishes in tributaries that flow into the Jurumirim Reservoir, such as the Taquarí and Guareí rivers.

The Guareí River has its mouth near the confluence between the Paranapanema River and the Jurumirim Reservoir (Leite et al. 2012). Few ichthyological studies have been conducted in this basin, and they have only investigated small watercourses (e.g., Castro et al. 2003b), lagoons (Seabra et al. 2012), population biology of two catfish species (Azevedo-Santos et al. 2018), and species description (Katz & Costa 2020), with no estimate of total diversity in the whole basin. Despite those studies, many tributary streams, as well as the Guareí River main channel, remain uninvestigated. In this sense, here we provide a broad survey of the fish species in the Guareí River basin, with the objective to provide the first report on fish diversity in this basin.

Material and Methods

1. *Study area*

The Guareí River is a tributary of the Paranapanema River, that flows into the upper section of the Jurumirim Reservoir (Leite et al. 2012). Its drainage catchment is entirely located in the State of São Paulo (Brazil), and covers the municipalities of Guareí, in the upper course, and Angatuba in the middle and lower portions (Fulan et al. 2012).

The drainage area is about 70,860 ha (Leite et al. 2012). Many tributary streams had their vegetation totally or partially removed, a trend observed in the whole basin. At the end of the last century, the Guareí River basin was composed mostly of pasture (> 50%) and recovered vegetation (~ 11%) (Henry & Gouveia 1993), with fragments of Mata Atlântica and Cerrado vegetation.

2. *Methodology*

We sampled fish between February 2017 and November 2018 (Table 1), under license number 57047 (SISBIO). We sampled the Guareí River main channel and more than 20 tributary streams of different orders (Figure 1), totaling 36 sites (i.e., channel segments) across the basin. We sampled more than one site in the Guareí River and in some of its main tributaries (e.g., Guarda-Mor stream) (Table 1).

Sampling in streams was carried out with a hand net (mesh ~ 1.5 mm), and when possible, cast net (mesh 14 mm) and two gill nets (mesh 10 mm and 20 mm) were employed. In streams, the hand net was operated in 50-100 m segments always in the upstream direction, during the day (between 7:00 am and 6:00 pm). In the Guareí River channel we employed gill nets with 30, 40, 50, 60, 70, 80 and 100 mm mesh size, which remained deployed overnight (~ 12 hours soak time). In addition, a castnet (mesh of 14 mm) and a hand net (mesh about 1.5 mm) were used, both methods directed to sample areas close to the margin.

Table 1. Information about the sites sampled across the Guareí River basin, Paranapanema River basin, São Paulo, Brazil.

SITE	NAME OF THE WATERBODIES	ORDER (*sensu* Strahler 1954)	COORDINATES	MUNICIPALITY	MONTH AND YEAR
1	Areia Branca stream	2	23°16'54.11"S, 48° 9'37.59"W	Guareí	August 2017 and May 2018
2	Unknown name	2	23°18'29.75"S, 48° 7'36.23"W	Guareí	August 2017 and May 2018
3	Inholava stream	3	23°20'49"S, 48°13'10.3"W	Guareí	March 2017 and May 2018
4	Unknown name	1	23°21'26.9"S, 48°12'49.3"W	Guareí	March 2017
5	Jacutinga da Boa Vista stream	3	23°22'56.8"S, 48°10'37.1"W	Guareí	August and December 2017
6	Guarda-Mor stream (lower course)	4	23°22'48.27"S, 48° 9'43.17"W	Guareí	February, August, and December 2017
7	Guarda-Mor stream (middle course)	3	23°24'28.41"S, 48° 8'41.84"W	Guareí	February and August 2017
8	Guarda-Mor stream (high course)	2	23°26'9.17"S, 48° 7'45.42"W	Guareí	August 2017 and May 2018
9	Grande stream (limit)	4	23°28'7.04"S, 48°15'8.24"W	Guareí-Angatuba	August 2017 and May 2018
10	Martinho stream	1	23°28'13.47"S, 48°19'24.59"W	Angatuba	June 2017 and June 2018
11	Unknown name	1	23°26'33.29"S, 48°19'42.93"W	Angatuba	April 2017
12	Durvalino stream	2	23°26'36.97"S, 48°20'40.80"W	Angatuba	April 2017 and May 2018
13	Unknown name	1	23°26'44.26"S, 48°21'35.16"W	Angatuba	April 2017 and June 2018
14	Unknown name	1	23°27'3.11"S, 48°22'13.19"W	Angatuba	April 2017 and May 2018
15	Unknown name	1	23°27'20.00"S, 48°22'25.51"W	Angatuba	April 2017 and May 2018
16	Unknown name	1	23°27'17.21"S, 48°23'19.17"W	Angatuba	April 2017 and June 2018
17	Corrente stream	2	23°26'05.7"S, 48°23'19.3"W	Angatuba	March, June, and September 2017
18	Cachoeira stream (below the waterfall)	2	23°27'36.28"S, 48°24'50.84"W	Angatuba	August 2017 and November 2018
19	Cachoeira stream (above the waterfall)	2	23°25'34.39"S, 48°25'13.24"W	Angatuba	February and September 2017
20	Guareí River channel (middle course)	-	23°27'53.90"S, 48°25'18.84"W	Angatuba	February and August 2017 and February, May, June and November 2018
21	Esperança stream	2	23°28'59.80"S, 48°25'7.27"W	Angatuba	February 2017 and June 2018
22	Catanduva stream (lower course)	3	23°29'18.12"S, 48°24'37.36"W	Angatuba	February 2017 and May 2018
23	Catanduva stream (high course)	1	23°31'12.05"S, 48°24'43.48"W	Angatuba	February 2017 and May 2018
24	Grande stream (middle course)	3	23°29'15.2"S, 48°24'28.2"W	Angatuba	April 2017 and May 2018
25	Grande stream (high course)	3	23°29'37.65"S, 48°20'8.98"W	Angatuba	February, April, June 2017 and November 2018
26	Unknown name	2	23°29'47.51"S, 48°20'18.38"W	Angatuba	February 2017 and May 2018
27	Libânios stream	3	23°27’34.4” S, 48°26’50.9” W	Angatuba	February 2017
28	Zacarias stream	3	23°29'21.8"S, 48°27'08.4"W	Angatuba	April 2017 and June 2018
29	Barra stream	3	23°29'25.62"S, 48°29'49.69"W	Angatuba	April 2017
30	Unknown name	1	23°29'28.78"S, 48°30'9.95"W	Angatuba	April 2017
31	Unknown name	2	23°29'14.74"S, 48°30'30.15"W	Angatuba	June 2017 and May 2018
32	Unknown name	1	23°29'6.57"S, 48°31'13.40"W	Angatuba	June 2017 and May 2018
33	Cambuí stream	3	23°28'24.31"S, 48°30'33.54"W	Angatuba	June 2017
34	Ribeiro stream	2	23°27'34.91"S, 48°29'42.72"W	Angatuba	June 2017
35	Aterradinho stream	3	23°27'33.77"S, 48°34'26.71"W	Angatuba	May 2018
36	Guareí River channel (lower course)	-	23°27'51.57"S, 48°34'40.57"W	Angatuba	June 2017 and January and February 2018

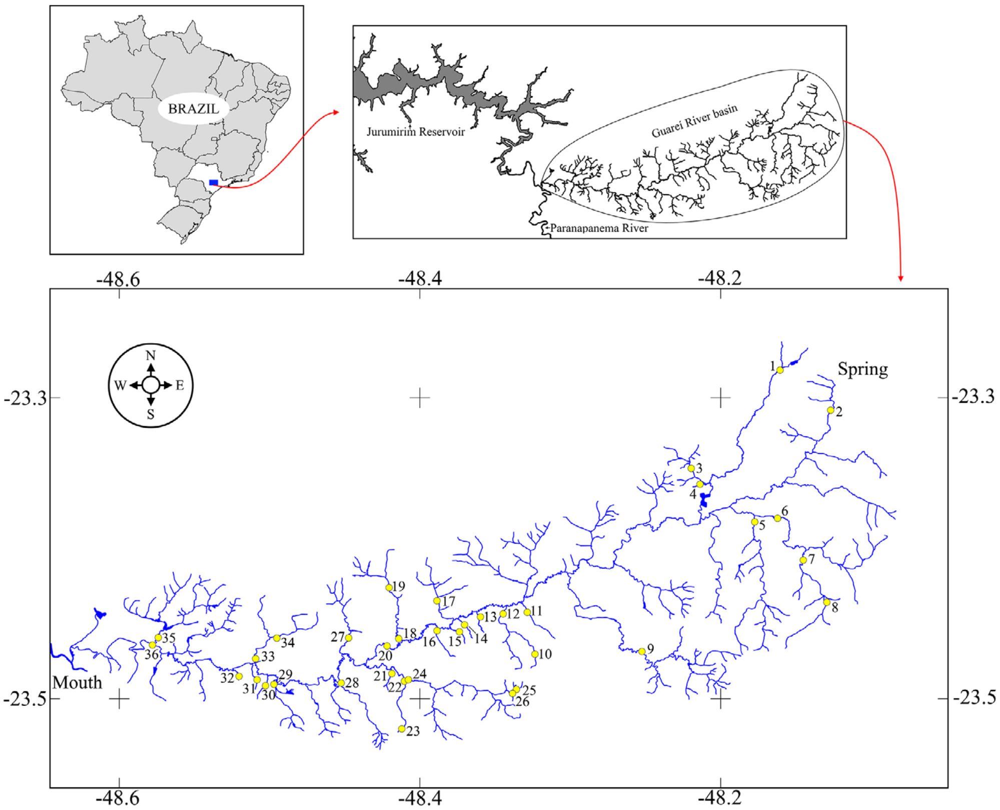

Figure 1. Guareí River basin and the location (yellow circles) of sampled sites (1 to 36). The map was drawn in the Software QGis (Sherman et al. 2012).

In the field, fishes were euthanized with a solution of water and eugenol or benzocaine (lethal dosage), and then preserved in formalin (10%). Voucher specimens were prepared (washed with water and transferred to 70% alcohol) and deposited in the following Brazilian biological collections: DZSJRP - Departamento de Zoologia e Botânica, Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), Campus de São José do Rio Preto, São Paulo; LBP - Laboratório de Biologia e Genética de Peixes, Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Botucatu, São Paulo; LIRP - Laboratório de Ictiologia de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, São Paulo; MNRJ - Museu Nacional, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Rio de Janeiro; NUP - Coleção Ictiológica do Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (Nupélia), Universidade Estadual de Maringá, Maringá, Paraná; UFRGS - Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul; UNT - Universidade Federal do Tocantins, Porto Nacional, Tocantins. Taxonomic classification followed Fricke et al. (2020a) for families, and Fricke et al. (2020b) for orders.

A rarefaction curve was calculated considering all species captured in the 36 sampled sites (based on Table S1 and Table S2). The figure was drawn in PRIMER 6 (Clarke & Warwick 2001).

Results

We captured 2,169 individuals belonging to 50 species in the Guareí River basin (Table 2; Figure 2 to Figure 6). The rarefaction curve for the studied area tended to stabilize, but the asymptote was not reached (Figure 7). These fishes belonged to 6 orders and 16 families, being Characiformes the most diverse, with 48% of all species (Figure 8). Characidae was the most diverse family (24%), followed by Loricariidae (14%) and Heptapteridae (12%) (Figure 9). Families with the lowest number of specie (with 2% each one) were Erythrinidae, Serrasalmidae, Trichomycteridae and Synbranchidae (Figure 9).

Local richness in each site ranged between 1 to 25 species. Sites 20 and 36, located in the Guareí River main channel, had 25 and 21 species respectively. Total abundance within sites ranged from 1 to 283 individuals (Table S1 e Table S2).

Astyanax lacustris (Lütken 1875) [Figure 3(e) and Figure S1(d)], a characid, was widely distributed in the studied area, in 21 sites. Other common species were *Psalidodon fasciatus* (Cuvier 1819)*,* found in 19 sites, and the catfish *Imparfinis mirini* Haseman 1911, present in 16 sites. The poecilid, *Phalloceros harpagos* Lucinda 2008, was the most abundant, with 253 individuals (Table 2), while *Leporinus friderici* (Bloch 1794), *L. striatus* Kner 1858, *Pimelodella gracilis* (Valenciennes 1835) and *Synbranchus marmoratus* Bloch 1795 were rare, with one individual each species.

Three species were non-native to the Guareí River basin, *Hyphessobrycon eques* (Steindachner 1882), *Oreochromis niloticus* (Linnaeus 1758) and *Poecilia reticulata* Peters 1859. Non-native species showed narrow distribution, being *H. eques* collected only in site 32, *O. niloticus* in site 29, and *P. reticulata* in sites 21 and 22, both located in the urban perimeter of the municipality of Angatuba. In general, non-natives were not abundant, excepting *P. reticulata* (cf. Table 2). The native status of *Gymnotus pantanal* Fernandes, Albert, Daniel-Silva, Lopes, Crampton, & Almeida-Toledo 2005, was uncertain (see Discussion section).

Table 2. Species (Actinopterygii) recorded in the Guareí River basin, Paranapanema River basin, São Paulo, Brazil.

SPECIES	**ABUNDANCE**	**STATUS**	**VOUCHER**
CHARACIFORMES			
Crenuchidae			
Characidium gomesi Travassos 1956	90	Native	MNRJ 50435
Characidium zebra Eigenmann 1909	7	Native	LBP 29211
Erythrinidae			
Hoplias malabaricus (Bloch 1794)	15	Native	LBP 29218
Parodontidae			
Apareiodon affinis (Steindachner 1879)	12	Native	LBP 29223
Parodon nasus Kner 1859	10	Native	LBP 29231
Serrasalmidae			
Serrasalmus maculatus Kner 1858	6	Native	LBP 29230
Anostomidae			
Leporinus friderici (Bloch 1794)	1	Native	DZSJRP 22777
Leporinus striatus Kner 1858	1	Native	LBP 29220
Megaleporinus obtusidens (Valenciennes 1837)	2	Native	LBP 29222
Schizodon nasutus Kner 1858	21	Native	LBP 29214
Curimatidae			
Cyphocharax modestus (Fernández-Yépez 1948)	56	Native	LBP 29228
Steindachnerina insculpta (Fernández-Yépez 1948)	33	Native	LBP 29221
Characidae			
Astyanax lacustris (Lütken 1875)	221	Native	DZSJRP 22817
Bryconamericus aff. *iheringii* (Boulenger 1887)	148	Native	DZSJRP 22812
‘*Cheirodon*’ *stenodon* Eigenmann 1915	23	Native	UFRGS 28084
Galeocharax gulo (Cope 1870)	8	Native	LBP 29229
Hyphessobrycon eques (Steindachner 1882)	3	Non-Native	LBP 29219
Oligosarcus paranensis Menezes & Géry 1983	6	Native	DZSJRP 22816
Piabarchus cf. *stramineus* (Eigenmann 1908)	24	Native	DZSJRP 22779
Piabina argentea Reinhardt 1867	11	Native	DZSJRP 22838
Psalidodon bockmanni (Vari & Castro 2007)	97	Native	DZSJRP 22747
Psalidodon fasciatus (Cuvier 1819)	166	Native	DZSJRP 22754
Psalidodon cf. *paranae* (Eigenmann 1914)	12	Native	DZSJRP 22757
Serrapinnus notomelas (Eigenmann 1915)	100	Native	DZSJRP 22756
GYMNOTIFORMES			
Gymnotidae			
Gymnotus carapo Linnaeus 1758	6	Native	LBP 29209
Gymnotus pantanal Fernandes, Albert, Daniel-Silva, Lopes, Crampton & Almeida-Toledo 2005	5	Indeterminate	LBP 29210
SILURIFORMES			
Trichomycteridae			
Cambeva guareiensis Katz & Costa 2020	71	Native	DZSJRP 22781
Callichthyidae			
Callichthys callichthys (Linnaeus 1758)	12	Native	LBP 29216
Hoplosternum littorale (Hancock 1828)	5	Native	LBP 29215
Loricariidae			
Hisonotus depressicauda (Miranda Ribeiro 1918)	26	Native	DZSJRP 22749
Hypostomus ancistroides (Ihering 1911)	26	Native	NUP 22311
Hypostomus iheringii (Regan, 1908)	62	Native	LBP 29213
Hypostomus sp. n.	57	Native	NUP 22310
Hypostomus tietensis (Ihering 1905)	2	Native	LBP 29212
Hypostomus strigaticeps (Regan 1908)	63	Native	NUP 22312
Rineloricaria pentamaculata Langeani & de Araujo 1994	7	Native	DZSJRP 22834

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Heptapteridae			
Imparfinis borodini Mees & Cala 1989	9	Native	DZSJRP 22741
Imparfinis mirini Haseman 1911	111	Native	LIRP 14330
Phenacorhamdia tenebrosa (Schubart 1964)	35	Native	DZSJRP 22752
Pimelodella avanhandavae Eigenmann 1917	37	Native	LBP 29226
Pimelodella gracilis (Valenciennes 1835)	1	Native	LIRP 14328
Rhamdia quelen (Quoy & Gaimard 1824)	9	Native	DZSJRP 22751
Pimelodidae			
Iheringichthys labrosus (Lütken 1874)	4	Native	LBP 29225
Pimelodus maculatus Lacepède 1803	7	Native	LBP 29224
SYNBRANCHIFORMES			
Synbranchidae			
Synbranchus marmoratus Bloch 1795	1	Native	DZSJRP 22827
CICHLIFORMES			
Cichlidae			
Geophagus brasiliensis (Quoy & Gaimard 1824)	60	Native	DZSJRP 22789
Oreochromis niloticus (Linnaeus 1758)	20	Non-Native	LBP 29217
CYPRINODONTIFORMES			
Poeciliidae			
Phalloceros harpagos Lucinda 2008	253	Native	UNT 016904
Phalloceros reisi Lucinda 2008	129	Native	UNT 16404
Poecilia reticulata Peters 1859	78	Non-Native	DZSJRP 22765

We collected two undescribed species, *Bryconamericus* aff. *iheringii* (Boulenger 1887) and *Hypostomus* sp. n. The first was widely distributed, while the second was recorded in the Guareí River and one stream (Table S1 and Table S2).

Discussion

This study recorded 50 fish species in the Guareí River basin. However, because the accumulation curve did not stabilize, other species may be found with further sampling effort. The Guareí River basin holds ~22% of all species richness known to the Paranapanema River basin (*sensu* Jarduli et al. 2020), an expressive value considering the relative small size of this catchment (~70.9 thousand ha; Leite et al. 2012). Moreover, the Guareí River basin hold ca. 16% of all species richness of the entire Upper Paraná River system (based on Langeani et al. 2007). These numbers and comparisons clearly indicate the importance of this tributary for the maintenance of fish diversity at multiple spatial scales (e.g., Guareí, Paranapanema and Paraná rivers). As a hotspot of fish diversity in the Upper Paranapanema River, the Guareí River basin needs adequate management and conservation actions to preserve its environmental integrity.

Characiformes and Siluriformes represented 84% of all species. Jarduli et al. (2020) asserted that these orders sum more than 70% of all species in the Paranapanema River basin. In the Guareí River basin, however, Characiformes dominated over Siluriformes, differing from the pattern observed in the Paranapanema River basin (*sensu* Jarduli et al. 2020). Characidae was the most diverse family in the studied area (24% of all species), followed by Loricariidae (14%). Cetra et al. (2016), studying streams in the Upper Paranapanema, found similar patterns, while Jarduli et al. (2020) recorded that both families hold 32% of all species in the Paranapanema River basin.

Almost all species (=46) are native to the Guareí River basin; only three were non-native and one indeterminate. This is an atypical trend in the Upper Paraná River, particularly the Paranapanema River, where several non-native species have been introduced (Garcia et al. 2018; Pelicice et al. 2018). The number of non-native species is also low when compared with areas close to the Guareí River basin, such as the Jurumirim Reservoir (Kurchevski & Carvalho 2014) and a tributary, the Taquari River (Nobile et al. 2017; Nobile et al. 2019).

The three non-native species are *Hyphessobrycon eques*, *Oreochromis niloticus* and *Poecilia reticulata*. Castro et al. (2003b) had previously registered *O. niloticus* in the Guareí River basin. Tilapias regularly escape from fish farms (Orsi & Agostinho 1999; Azevedo-Santos et al. 2011; Casimiro et al. 2018), and a fish farm is located upstream from the site where *O. niloticus* was collected. The characid *H. eques* is widely distributed in the Upper Paraná River, and some authors suggest that it is native to this watershed (Langeani et al. 2007). However, numerous other studies indicate that the species was introduced into the Paranapanema River basin (e.g., Kurchevski & Carvalho 2014; Vidotto-Magnoni et al. 2015; Pelicice et al. 2018; Jarduli et al. 2020). We agree that it is non-native to the Guareí River basin. *Poecilia reticulata* is widespread in southeastern Brazil (Dias et al. 2020), including the Upper Paraná basin (e.g., Araújo et al. 2011; Cunico et al. 2019; Pagotto et al. 2012; Alves et al. 2016). In our study, this fish was captured in sites with urban impact, within the city of Angatuba. Mosquito control is probably the cause of its introduction, as this species has been commonly used to control *Aedes* populations (Azevedo-Santos et al. 2016).

Figure 2. Some fish species (Actinopterygii) collected in the Guareí River basin: (a) *Characidium gomesi*, 51.5 mm SL; (b) *Characidium zebra*, 64.8 mm SL; (c) *Hoplias malabaricus*, 93.5 mm SL; (d) *Apareiodon affinis*, 89.7 mm SL; (e) *Parodon nasus*, 91.3 mm SL; (f) *Serrasalmus maculatus*, 86. 7 mm SL; (g) *Leporinus friderici*, 144.9 mm SL; (h) *Leporinus striatus*, 87.1 mm SL; (i) *Megaleporinus obtusidens*, 86.2 mm SL; (j) *Schizodon nasutus*, 137.2 mm SL.

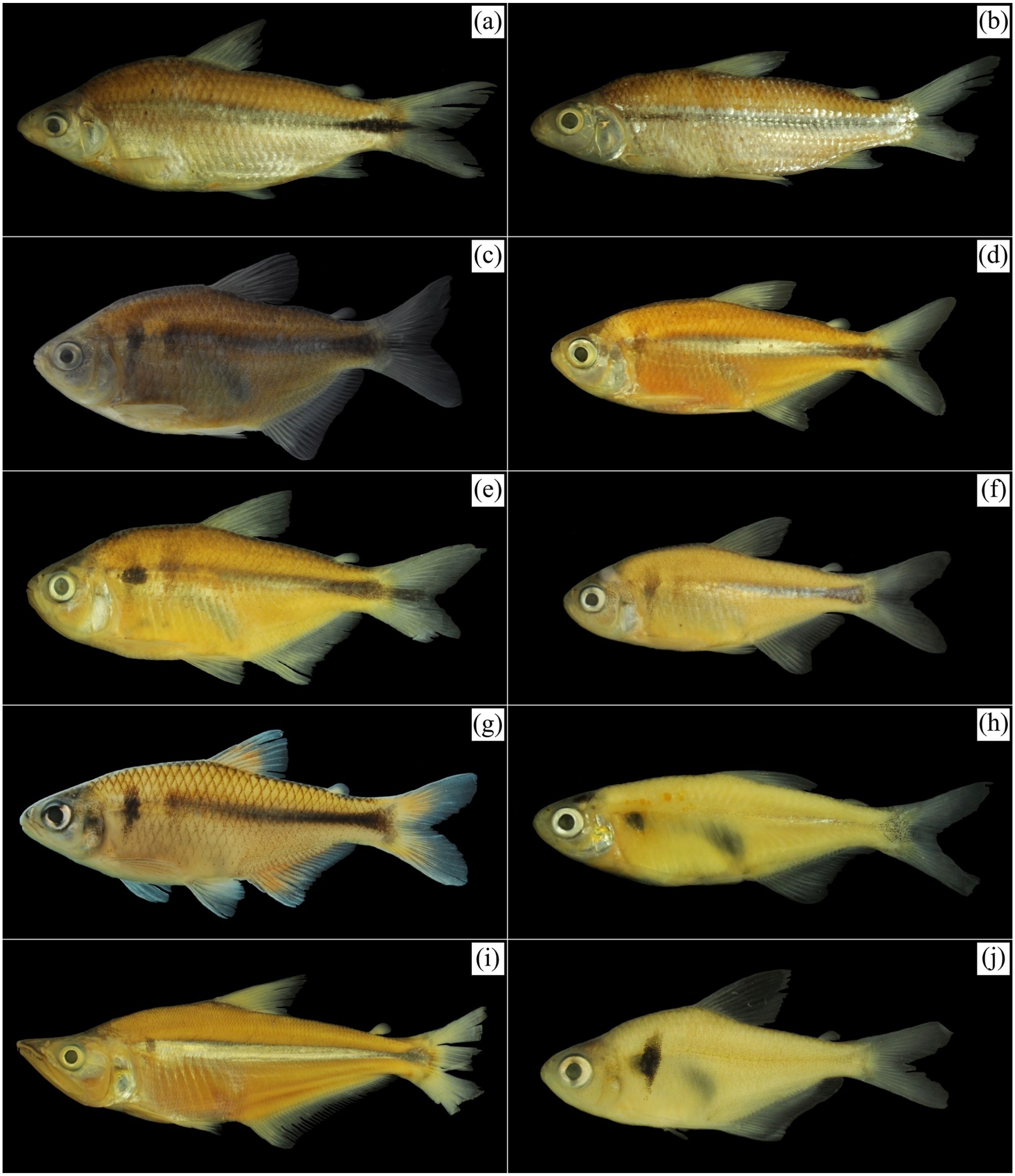

Figure 3. Some fish species (Actinopterygii) collected in the Guareí River basin: (a) *Cyphocharax modestus*, 96.4 mm SL; (b) *Steindachnerina insculpta*, 67.9 mm SL; (c) *Psalidodon bockmanni*, 59.8 mm SL; (d) *Psalidodon fasciatus*, 63.1 mm SL; (e) *Astyanax lacustris*, 64.3 mm SL; (f) *Psalidodon* cf. *paranae*, 38.1 mm SL; (g) *Bryconamericus* aff. *iheringii*, 49.1 mm SL; (h) '*Cheirodon*' *stenodon*, 29.1 mm SL; (i) *Galeocharax gulo*, 167.9 mm SL; (j) *Hyphessobrycon eques*, 22.1 mm SL.

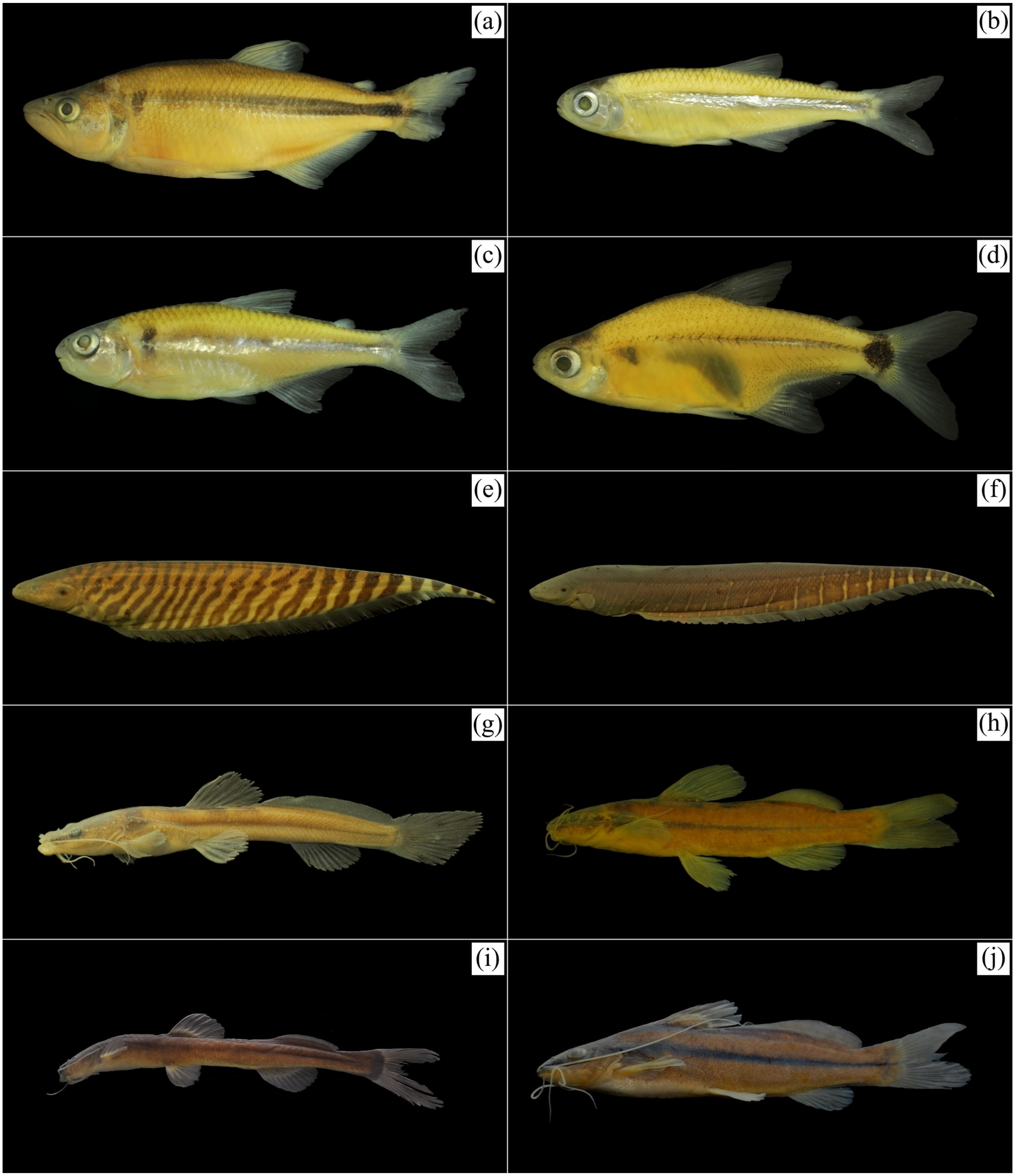

Figure 4. Some fish species (Actinopterygii) collected in the Guareí River basin: (a) *Oligosarcus paranensis*, 144.3 mm SL; (b) *Piabarchus* cf. *stramineus*, 48.1 mm SL; (c) *Piabina argentea*, 58.2 mm SL; (d) *Serrapinnus notomelas*, 29.5 mm SL; (e) *Gymnotus carapo*, 108.6 mm TL; (f) *Gymnotus pantanal* 197.2 mm TL; (g) *Imparfinis borodini*, 32.8 mm SL; (h) *Imparfinis mirini*, 60.2 mm SL; (i) *Phenacorhamdia tenebrosa*, 80.1 mm SL; (j) *Pimelodella avanhandavae*, 83.7 mm SL.

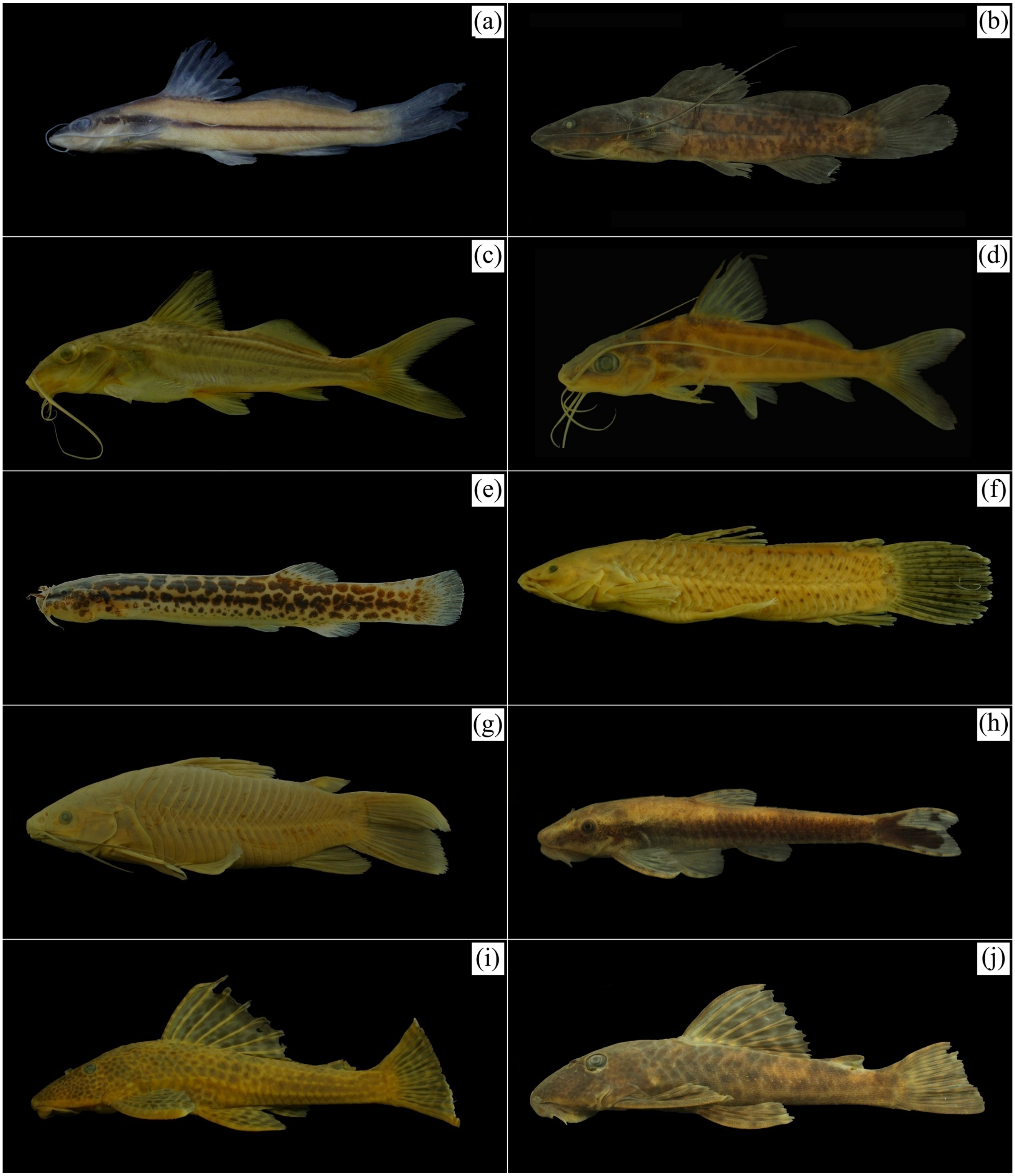

Figure 5. Some fish species (Actinopterygii) collected in the Guareí River basin: (a) *Pimelodella gracilis* 60.1 mm SL; (b) *Rhamdia quelen*, 47.6 mm SL; (c) *Iheringichthys labrosus*, 101.8 mm SL; (d) *Pimelodus maculatus*, 81.9 mm SL; (e) *Cambeva guareiensis*, 55. 4 mm SL; (f) *Callichthys callichthys*, 56.5 mm SL; (g) *Hoplosternum littorale*, 100.8 mm SL; (h) *Hisonotus depressicauda*, 34.8 mm SL; (i) *Hypostomus ancistroides*, 141.4 mm SL; (j) *Hypostomus iheringii*, 93.7 mm SL.

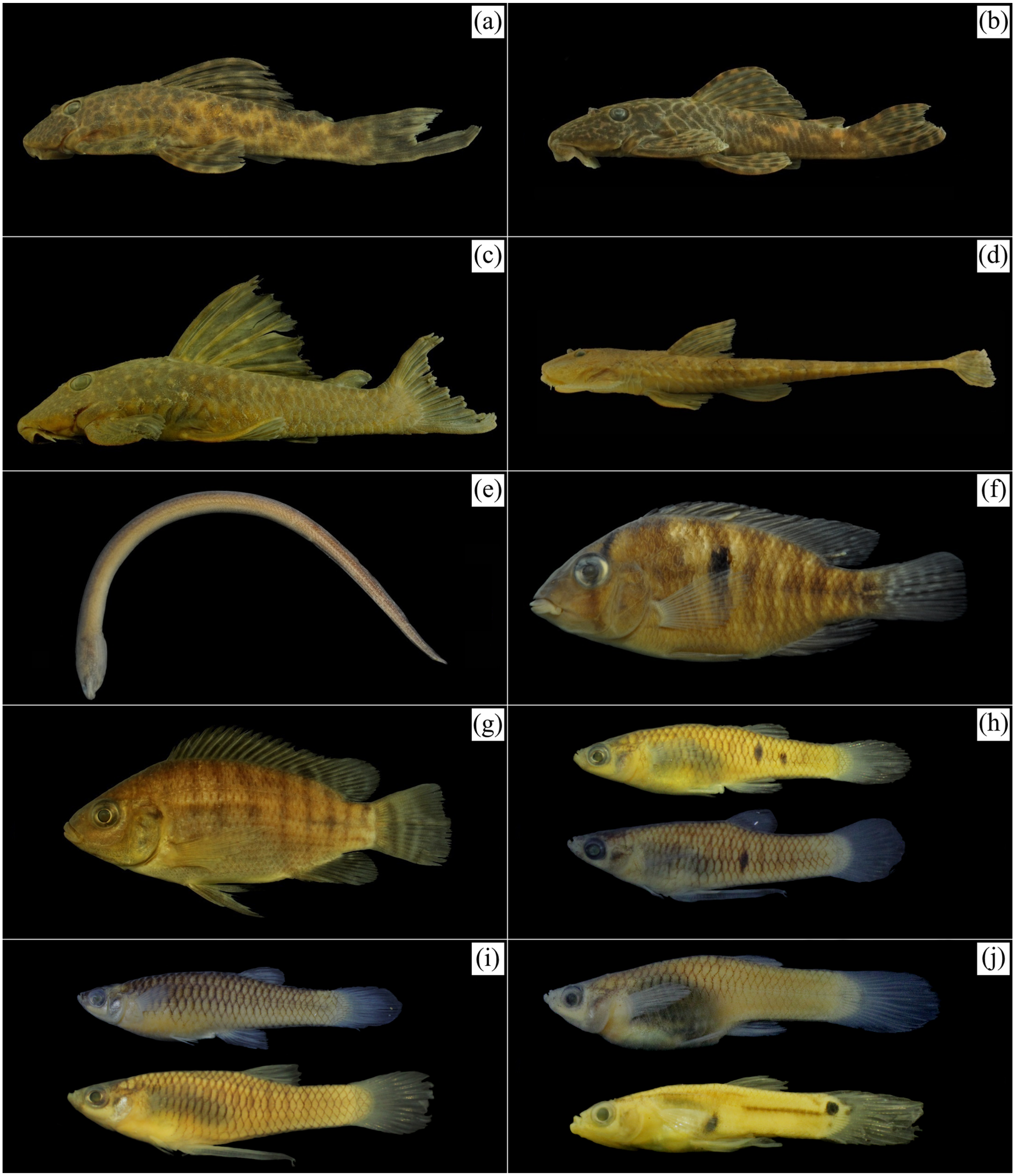

Figure 6. Some fish species (Actinopterygii) collected in the Guareí River basin: (a) *Hypostomus* sp. n., 81.3 mm SL; (b) *Hypostomus tietensis*, 81.5 mm SL; (c) *Hypostomus strigaticeps*, 129.4 mm SL; (d) *Rineloricaria pentamaculata*, 145.3 mm SL; (e) *Synbranchus marmoratus*, 131.7 mm TL; (f) *Geophagus brasiliensis*, 58.7 mm SL; (g) *Oreochromis niloticus*, 64.4 mm SL; (h) *Phalloceros harpagos*, 24.5 mm SL (female above), 16.3 mm SL (male below); (i) *Phalloceros reisi*, 40.7 mm SL (female above), 25.4 mm SL (male below); (j) *Poecilia reticulata*, 24.9 mm SL (female above), 16.4 mm SL (male below).

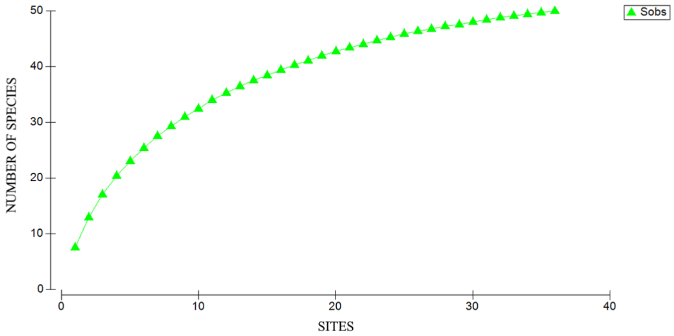

Figure 7. Species accumulation curve based on sampling effort in the Guareí River basin, Paranapanema River, São Paulo, Brazil.

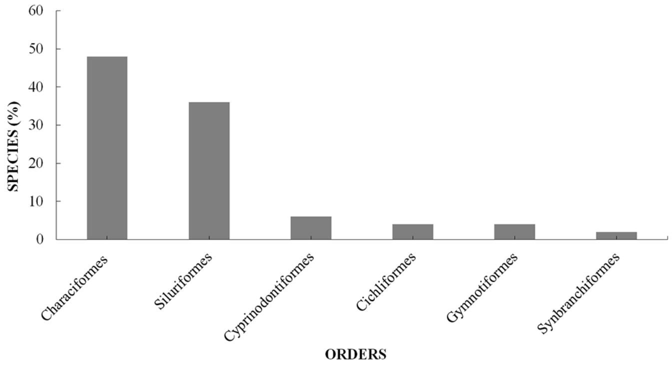

Figure 8. Species richness (%) in each taxonomic order found in the Guareí River basin, Paranapanema River, São Paulo, Brazil.

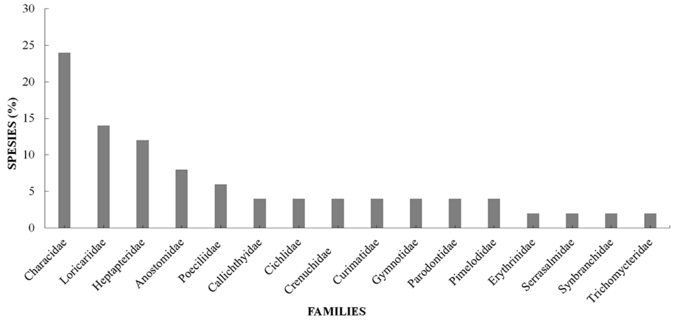

Figure 9. Species richness (%) in each taxonomic family found in the Guareí River basin, Paranapanema River, São Paulo, Brazil.

The natural origin of the electric eel *Gymnotus pantanal* is uncertain. This species was described by Fernandes et al. (2005), with occurrence to the lower portion of the Paraná River, the La Plata River, and a tributary of the Amazon basin. Some authors (Júlio Júnior et al. 2009; Jarduli et al. 2020) argued that *G. pantanal* reached the Upper Paraná after the flooding of Sete Quedas waterfalls (after the construction of Itaipu Dam), which worked as natural barrier between the Upper and Middle Paraná basins. It is unlikely, however, that the loss of Sete Quedas caused this introduction in the Guareí River basin, considering the distance and the number of dams along the Paranapanema River (cf. Pelicice et al. 2018), most of them built before Itaipu (e.g., Jurumirim). Alternatively, sport fishing has been an important vector causing the introduction of non-native species in Brazilian waters (e.g., Daga et al. 2016; Pereira & Vitule 2019), and could be responsible for the introduction of *G. pantanal* as live baits (Jarduli et al. 2020). However, it would need repeated events of introduction in the Guareí River basin, which seems unlikely. Therefore, the status of *G. pantanal* in the Guareí River basin deserves further examination.

We recorded three "migratory" species (*sensu* Vazzoler 1996: p. 96-97), *Leporinus friderici*, *Megaleporinus obtusidens* (Valenciennes 1837), and *Pimelodus maculatus* Lacepède 1803. These fishes provide important income for fishermen living in the Angatuba region (Novaes & Carvalho 2009) and other cities along the Paranapanema River. Other migratory fishes are found in the Jurumirim Reservoir and tributaries, such as *Prochilodus lineatus* (Valenciennes 1837) and *Salminus hilarii* Valenciennes 1850 (Kurchevski & Carvalho 2014; Nobile et al. 2019). They may occur in the Guareí River basin, particularly in the main channel, either year-round or seasonally, using this tributary as a feeding ground or spawning site.

In the description of *Phalloceros reisi*, Lucinda (2008) recorded the species for the upper Paraná River system, but only for waterbodies of the Tietê River basin. Cetra et al. (2012) were probably the first to record this species in the Paranapanema River basin. Later, other authors reported its presence in different waterbodies of the same watershed (Cerqueira et al. 2016; Cetra et al. 2016; Peressin et al. 2018). In this study, we found *P. reisi* in 12 sites, expanding its range in the Paranapanema River basin.

We also found two undescribed taxa, *Bryconamericus* aff. *iheringii* and *Hypostomus* sp. n. Both had already been captured in areas close to the Guareí River basin (Kurchevski & Carvalho 2014), and the present work expanded their known range. Herein, we just mention that the description of *Hypostomus* sp. n. is in progress (C. H. Zawadzki, pers. comm.). *Cambeva guareiensis* Katz & Costa 2020, a recently described species, is known only from streams in the Guareí River Guareí River basin (Katz & Costa 2020; this work). As observed for other species of the genus (e.g., *Cambeva diabola* Bockmann, Casatti, de Pinna 2004), it may have a wider distribution.

The identification of some species was uncertain. *Psalidodon* cf. *paranae* (Eigenmann 1914) integrates the complex '*Astyanax*' *scabripinnis*, which presents similar morphology (Bertaco & Lucena 2006), including undescribed species (e.g., Azevedo-Santos et al. 2019). Until a better definition of *P. paranae*, we treat the form captured in the Guareí River basin with caution. On the other hand, *Piabarchus* cf. *stramineus* (Eigenmann 1908) shows no trace of chromatophores in the humeral region, as reported by Eigenmann (1908) in the original description of this species. It is not known whether this character represents intraspecific variation, so we also treat its identification with caution.

Conclusion

This is the most comprehensive survey on fish diversity in the Guareí River basin. We recorded 50 species, most of which are native to the watershed, including migratory fishes and some taxa unknown to science. Fish diversity may be greater, considering the trend observed in the rarefaction curve. These results emphasize the role of tributaries as hotspots of fish biodiversity in impounded areas, with an important function in reservoirs that support fishing activity, such as the Jurumirim Reservoir. Therefore, conservation efforts must be directed to the Guareí River basin, especially to protect its tributaries.

Supplementary Material

The following online material is available for this article:

Figure S1 – Individuals (in life) of 15 species collected from the Guareí River basin: (a) *Characidium gomesi*; (b) *Hoplias malabaricus*; (c) *Psalidodon bockmanni*; (d) *Astyanax lacustris*; (e) *Bryconamericus* aff. *iheringii*; (f) *Serrapinnus notomelas*; (g) *Gymnotus carapo*; (h) *Phenacorhamdia tenebrosa*; (i) *Rhamdia quelen*; (j) *Callichthys callichthys*; (k) *Hisonotus depressicauda*; (l) *Rineloricaria pentamaculata*; (m) *Geophagus brasiliensis*; (n) *Oreochromis niloticus*; (o) *Phalloceros reisi*.

Table S1 – Species (Actinopterygii) collected and their abundance in each site (1 to 18) of the Guareí River basin, São Paulo, Brazil.

Table S2 – Species (Actinopterygii) collected and their abundance in each site (19 to 36) of the Guareí River basin, São Paulo, Brazil.

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Author Contributions

Valter M. Azevedo-Santos idealized the work, collected and identified species, provided ideas, wrote the first version of the manuscript.

Fernando M. Pelicice provided ideas and collaborated with the writing process.

Raoul Henry provided ideas and collaborated with the writing process.

Conflicts of Interest

None.

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Article

How oogenesis analysis combined with DNA barcode can help to elucidate taxonomic ambiguities: a polychaete study-based approach

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Abstract: Polychaetes are common in coastal and estuarine environments worldwide and constitute one of the most complex groups of marine invertebrates. The morpho-physiology of the female reproductive system (FRS) can be understood by using histological tools to describe reproductive cycle and gametogenesis paths and, among other purposes, aiming to identify and differentiate polychaete species. However, this histology-based approach is rarely combined with molecular tools, which is known to accurately delimitate species. In the same way, the description and understanding of oogenesis and vitellogenesis paths within polychaetes are lacking for most families, narrowing the range of its utility. Therefore, the present study aims to describe the oogenesis in three polychaete species common and abundant on the South American Atlantic coast (*Laeonereis culveri, Scolelepis goodbodyi* and *Capitella biota*) and investigate the utility of reproductive features and gametogenesis as a relevant associate knowledge to discriminate species, particularly useful for putative cryptic species, integrated with morphological and molecular data. In a first attempt, the results obtained herein allow the authors to describe two new subtypes of oogenesis, dividing it in extraovarian oogenesis type I and II and intraovarian type I and II. The results also demonstrate that the following histological characters of the FRS can be relevant for the separation of related species: a) oogenesis type, b) occurrence or absence of a true ovary, c) ovary tissue organization, d) type of accessory cells present, and e) oocyte morphology. Additionally, these histological features of FRS, when compared with correlated species studied under this scope, converge with the genetic data. The analysis of cytochrome oxidase I (COI) barcode sequences differentiates between North and South American Atlantic populations of *L. culveri* (16.78% genetic distance), while in S. *goodbodyi* and *C. biota* it discriminates them from their congeneric species. These results highlight the importance of multi-tool approach and shows that both FRS histology and histo-physiology, and DNA barcoding can be used to identify and discriminate cryptic species, which is usually not possible when using morphological characters. Besides, these characters may also be useful in differentiating related species, and/or geographically distinct populations among polychaetes.

Keywords: *Integrative taxonomy; "Polychaeta"; oogenesis; histology; COI; cryptic species.*

Como análises de oogênese combinadas com DNA barcode podem elucidar ambiguidades taxonômicas: uma abordagem baseada em estudos com poliquetas

Resumo: Os poliquetas são comuns em ambientes costeiros e estuarinos em todo o mundo e constituem um dos grupos mais complexos de invertebrados marinhos. A morfo-fisiologia do sistema reprodutor feminino (FRS) pode ser compreendida por meio de ferramentas histológicas para identificar e diferenciar estes anelídeos. No entanto, essa abordagem histológica raramente é combinada com ferramentas moleculares, amplamente conhecidas por delimitar espécies congenéricas ou crípticas com maior precisão. Do mesmo modo, a descrição e o entendimento da oogênese e vitelogênese dentre os poliquetas, para a maioria das famílias, é ainda limitado. Portanto, o presente estudo tem como objetivo descrever a oogênese em três espécies de poliquetas comuns e abundantes na costa sul-americana (*Laeonereis culveri, Scolelepis goodbodyi e Capitella biota*) e investigar a utilidade das características reprodutivas e da gametogênese como um conhecimento associado relevante para discriminar espécies, particularmente útil para espécies crípticas putativas, integradas a dados morfológicos e moleculares. Os resultados aqui obtidos permitiram descrever dois novos subtipos de oogênese, dividindo-a em oogênese extra-ovariana dos tipos I e II e intra-ovariana dos tipos I e II. Os resultados também demonstram que os seguintes caracteres histológicos do FRS podem ser relevantes para a separação de espécies relacionadas: a) tipo de oogênese, b) presença ou ausência de um ovário verdadeiro, c) organização tissular ovariana, d) tipo de células acessórias presentes e, e) morfologia do ovócito. Além disso, essas características histológicas do FRS, quando comparadas às espécies correlatas estudadas sob esse escopo, convergem com os dados genéticos separando espécies putativas e congenéricas. As análises com DNA barcode demonstraram que em *L. culveri* é possível diferenciar as populações atlânticas Norte e Sul-americanas (16,78% de distância genética), enquanto para *S. goodbodyi* e *C. biota* fica evidente sua distinção com espécies congenéricas. Esses resultados destacam a importância da abordagem com múltiplas ferramentas e mostram que tanto a histologia quanto a histo-fisiologia do FRS e o DNA barcode podem ser usados para identificar e discriminar espécies crípticas e potencialmente crípticas, o que geralmente não é possível quando se utilizam apenas caracteres morfológicos. Além disso, esses caracteres também podem ser úteis na diferenciação de espécies relacionadas e / ou populações geograficamente distintas desses poliquetas.

Palavras-chave: *Taxonomia integrativa; "Polychaeta"; oogênese; histologia; COI; espécies crípticas.*

Introduction

Polychaetes reproduce mainly sexually, however asexual reproduction is commonly found within the group. Both forms present a great diversity of reproductive and developmental modes, in which the reproductive system's morphology itself, and oogenesis and vitellogenesis paths, display relevant characters for a broad range of research applications (Schroeder & Hermans 1975, Eckelbarger 2001, Rouse & Pleijel 2001, Aguado et al. 2014). Among individuals that reproduce sexually, the majority is dioecious and presents a very simplified reproductive system when compared to other invertebrates. In some cases, there are no tissues and / or organs specialized in the production of germ cells, in its storage or transportation to other body regions and even to the external environment. On the other hand, some species such as *Bathykurila guaymasensis* Pettibone 1989 and other deep-sea annelids show a complex and well tissue-organized reproductive system (Schroeder & Hermans 1975, Eckelbarger 2001, Rouse & Pleijel 2001, Glover et al. 2005, Aguado et al. 2014, Faroni-Perez & Zara 2014).

In polychaetes, oogenesis occurs in two distinct stages: the proliferative phase, in which the oogonia duplicates by mitosis; and the growth phase, in which oogonia I (pre-meiotic) and oogonia II (pre-vitellogenic) go through meiosis and initiate the maturation process (hypertrophy and vitellogenesis). In some species, the oogonia II exhibits cytoplasmic bridges between two or more cells at the end of the meiosis, while in others these bridges are observed in the early stages of growth phase (oocytes) (Adiyodi & Adiyodi 1983, Eckelbarger 2005). Among the studied species to date, two basic forms (types) of oogenesis were described: **a)** intraovarian, where oocytes develop completely within an ovary, associated or not with follicular cells; and **b)** extraovarian, in which the final differentiated oogonia or the immature oocytes detach from their proliferative tissue and reach the coelomic cavity, where it conclude development (Wilson 1991, Eckelbarger 1994, 2001, 2005).

The histo-physiological variations found in gametogenesis can provide important information on how these reproductive modes evolved among invertebrate taxa, especially in Annelida (Rouse & Pleijel 2001), which is of great value for evolutionary and taxonomic studies. In the same way, reproductive and gametogenesis features within polychaetes have been identified as useful for construction of phylogenetic hypothesis, such as vitellogenesis paths and/or oocyte morphology (Faroni-Perez & Zara 2014). Furthermore, studies regarding polychaetes gametogenesis have been done in only 0.1% of the described species (Eckelbarger 2005), and in the Americas these studies are restricted to few species that are ecologically relevant for environmental monitoring, usually pertaining the life-history or reproductive cycle (Eckelbarger 2005, MacCord & Amaral 2007, Garraffoni et al. 2014).

Faroni-Perez & Zara (2014) and Nunes et al. (2017) performed histochemical, ultrastructural and phylogeographic studies, in a complementary way, showing evidence of intraspecific variation in reproductive features on a presumed cosmopolitan species along the Atlantic waters, *Phragmatopoma lapidosa* Kinberg, 1866, and molecular evidence confirming the existence of two distinct species between North Western and South Western Atlantic regions.

Glover et al. (2005) and Meiβner & Götting (2015) also used histological features to describe and delimitate annelid species; the first elucidate reproductive characteristics of *B. guaymasensis* common with congeneric species and with other hydrothermal vent annelids, while the second presents histological differences in tissue composition in the ventral epidermal glands of representative Spionidae from Australia. Meanwhile, other species, such as *Laeonereis culveri* (Webster 1879) (Nereididae), *Scolelepis goodbodyi* (Jones 1962) (Spionidae) and *Capitella biota* Silva & Amaral 2017 (in Silva et al. 2017) (Capitellidae), which are common and very abundant in tropical and subtropical Atlantic shallow waters and/or intertidal zones of South American coasts, have not been studied under this scope (Omena & Amaral 2001, MacCord & Amaral 2007, Oliveira 2009, Silva et al. 2017).

The use of molecular tools like DNA barcoding for specimen identification and classification has been shown to be successful in several marine groups (Radulovici et al. 2009, Knebelsberger et al. 2015, Raupach et al. 2015). Its usage has become quite widespread in marine invertebrates, often as a complement to morphological identifications and providing a quick screening method for highlighting mismatching morphological and molecular data, and detect putative cryptic species, species complexes, and inaccurate or misleading identifications (Hebert et al. 2003, Hajibabaei et al. 2006, Costa & Antunes 2012, Lobo et al. 2016). In this way, integrative approaches encompassing one or more morphological analyses (i.e. histology, transmission electron microscopy, scanning electron microscopy) with molecular data, namely DNA barcodes, are desirable and encouraged for a better taxonomic resolution (Langeneck et al. 2020; Martin et al. 2020; Teixeira et al. 2020)

Given this scenario, this study aims to describe female gametogenesis in these three species of polychaetes, bringing to light new data regarding oogenesis of this group and investigate the utility of histology as a relevant associate tool to discriminate species, particularly useful for putative cryptic species, which are supposed to be distinguishable through molecular methods only. Considering the apparently high incidence of cryptic species among polychaetes (Nygren 2014, Lobo et al. 2016), we anticipate that the application of histological and histo-physiological analysis will be particularly relevant for the taxonomy and systematics of this highly diverse group of invertebrates.

Material and Methods

1. Collection of specimens

The following species were analyzed: *Laeonereis culveri* (20 specimens), *Scolelepis goodbodyi* (20 specimens), both collected in Araçá Bay, São Sebastião, Brazil (23º48'49.9"S 45º24'31.3"W), during the summer months of 2016; and *Capitella biota* (five specimens), collected at Praia do Perequê, Guarujá, Brazil (23º56'31.0''S 46º10'25.3''W), in the early fall of 2017. The analyzed material was collected manually with a shovel in days of low syzygy tides. These species were studied for four reasons: **1)** they are abundant in sandy and muddy bottoms of the intertidal region; **2)** belong to distinct families; **3)** in the case of *L. culveri*, taxonomic or identification ambiguities/issues are reported; and **4)** reproductive cycles of congeneric species are documented in the literature, hence allowing comparisons.

2. Histology

For the histological analysis, at least five ovigerous females of each species were chosen by the observation of oocytes in their coelomic cavity and fixed in 10% glutaraldehyde solution in Phosphate Saline Buffer (PBS) with addition of 7% sucrose. After a minimum of 72 h below 4 ºC, the subjects were washed in PBS for 5 min and photographed using a stereomicroscope (Zeiss Axio Zoom Imager M2). Some of the ovigerous females were dissected to release the oocytes from the body cavity to describe their external morphology.

The same individuals were then dehydrated in 70, 80, 90 and 95% ethanol for 15 min at each concentration. Following the dehydration, infiltration with embedding historesin was performed for at least seven days, and then the final inclusion in Leica historesin for blocks polymerization was conducted. Each block was sectioned in microtome (Leica RM2245) in slices of 3.5 µm each, collected on glass slides and stained with Harris - eosin hematoxylin for further photo documentation under light microscopy (Zeiss Axio Imager M2). A total of 18 slides were analyzed for each species.

3. DNA extraction and amplification

Five *L. culveri* and six *S. goodbodyi* specimens both sampled from the Araçá Bay were fixed in ethanol 99% and used in subsequent molecular analysis. The only exception was *C. biota*, because cytochrome oxidase I (COI) barcode sequences from this species were already available from the same sampling location (Silva et al. 2017) and the specimens were confirmed by the authors.

DNA extraction was performed using the E.Z.N.A. Mollusc DNA Kit (Omega Bio-tek) according to manufacturer instructions. A small amount of tissue of each specimen was used. Then, the 658-base pair (bp) fragment from the 5'end of COI was amplified using the set of primers PolyLCO/PolyHCO (Carr et al. 2011). All PCR reactions were performed in a 25 µl volume containing 2.5 µl of 10X PCR buffer + KCl, 2.5 µl of 25 mM MgCl2, 0.5 µl of 10 mM dNTPs, 0.2 µl of Taq polymerase (Thermo Fischer Scientific) and 1.5 µl of each primer (10 mM). DNA template varied between 2 µl and 4 µl. Cycling conditions for PCR reactions with the primer pair PolyLCO/PolyHCO were: one cycle of 94 ºC for 1 min, 5 cycles of 94 ºC for 40 s, 45 ºC for 40 s and 72 ºC for 60 s, 35 cycles of 94 ºC for 40 s, 51 ºC for 40 s and 72 ºC for 60 s, with a final extension of 72 ºC for 5 min. Amplification success was checked in a 1.5% agarose gel, using 5 µl of PCR product, and successful PCR products were then purified (ExoSAP protocol - Thermo Fisher Scientific). Cleaned-up amplicons were sent to external sequencing service suppliers (Macrogen Europe, Spain), for bidirectional sequencing.

The sequences obtained for *L. culveri* and *S. goodbodyi* in the present study were deposited in BOLD under the dataset "SCLAE" DOI: dx.doi.org/10.5883/DS-SCLAE. All sequences are also available at the GenBank and accession number is provided for each sequence mined from database in phylogenetic trees presented herein.

4. Genetic analysis and data treatment

All sequences were analyzed and edited using MEGA 7.0 (Kumar et al. 2016). Trace files were checked manually, unreadable zones and primers removed, and ambiguous bases corrected.

Then, the edited sequences were aligned using Clustal W (Thompson et al. 1994) implemented in MEGA 7.0 (Kumar et al. 2016) and the translation verified for stop codons or indels. GenBank BLASTn search (Altschul et al. 1990) and BOLD Identification System tool (Ratnasingham & Hebert 2007) were used to search for similarity to confirm the target taxa.

When publicly available (in GenBank and/or BOLD), representative COI sequences of the same or congeneric species were added to the genetic analysis. Sequences publicly available that raised uncertainty regarding their confidence were excluded. To assure this proofreading, two steps were applied. First, all available sequences were pre-screened for codon-stops, indels, sequence length (more than 500 bp) and ambiguous or incomplete taxa names. Then, a second step was done to evaluate misidentifications by constructing a preliminary neighbor-joining tree in MEGA 7.0 (Kumar et al. 2016) using Kimura-2-parameter model (1×10^3 bootstraps of support). Whenever distinct taxa clustered together, the more represented taxa or the ones associated to a peer-reviewed publication were accepted.

Interspecific (or between geographic distant populations) distances were calculated using pairwise distances (1000 bootstraps replicates) in MEGA 7.0 (Kumar et al. 2016). Maximum likelihood (ML) trees for COI were constructed based on the best-fitting model of nucleotide substitution implemented in MEGA 7.0 (Kumar et al. 2016) for each group: GTR+G+I (for *L. culveri* and *S. goodbodyi)* and HKY+G (for *C. biota*).

Results

1. *Morphology of reproductive characters*

Table 1 shows a summary of the FRS morphological and histological features obtained through the comparative oogenesis analysis of the studied polychaete species.

1.1. Laeonereis culveri (Figure 1A-C)

In this species, it is possible to observe oocytes through the specimen tegument floating in the coelom (Figure 1B). They are large, spherical cells and occur in varying numbers according to the female's reproductive stage. In *L. culveri* females that are in an early stage of gametogenesis, primordial germ cell clusters can be observed in some setigers, presented as a white spherical "spot" when observed with naked eye. Under stereomicroscope, this cluster is most clearly seen with a lobed shape (Figure 1C).

1.2. Scolelepis goodbodyi (Figure 1D-F)

In *S. goodbodyi* a different organization and distribution of germ cells and their original tissues can be observed. No free-floating oocytes were observed in the specimen's coelomic cavity, but oval yellowish oocytes packaged in the parapodia of each setiger after the 24th or 25th segment (Figure 1E). Inside the germinal setiger a sac-like tissue is observed, a sheath that houses the developing germ cells, trapped in the specimen coelomic wall (Figure 1E). The oocytes in advanced stage of vitellogenesis are elliptic cells with an oval and centralized germinal vesicle (Figure 1F). The oocytes surface is very rich in membrane specializations (villi) which form a honeycomb-like net, just below which a thin darker line is observed suggesting that the deposition of the shell begins at the end of the vitellogenesis and just below these villi (Figure 1F).

1.3. Capitella biota (Figure 1G-I)

In this species, some organization was also observed in the distribution of oocytes in the coelom, in which the occurrence of oocytes from the fifth setiger of the specimen can be noticed, arranged in pairs in each segment and not more than six in number. They are large, elliptic cells with round germinal vesicles (mature oocytes) (Figure 1H and I), showing some disproportionality regarding the dimensions of the adult animal. The coelom is full of follicular cells that appear in clusters or individually (Figure 1H).

2. *Histology*

The following germ cells were observed at different developmental stages: **a)** primary oocytes (**poo**), cells in the pre-meiotic stage (interphase) or onset of meiosis II; **b)** secondary oocytes (**soo**), end-stage meiosis II cells; and **c)** mature oocytes (**Oo**) showing different morphological features due to vitellogenesis process.

Table 1. List of relevant histological characters of the female reproductive system for each species studied. (1) = species studied in this work; (2) = data obtained from Klesch (1970); (3) = data obtained from Richards (1970); (4) = data obtained from Eckelbarger & Grassle (1982, 1983). (*) Differences between species observed in the present study.

	SPECIES					
CHARACTERS	***L. culveri* (1)**	***L. culveri* (2)**	***S. goodbodyi* (1)**	***S. squamata* (3)**	***C. biota* (1)**	***C. teleta / C. jonesi* (4)**
Oogenesis type	Extraovarian type II (*)	Extraovarian type I (*)	Intraovarian type II (*)	Intraovarian type I (*)	Intraovarian type II	Intraovarian type II
Ovary organization and location	Lack	Lack	Paired organ in each setiger	Paired organ in each setiger	Paired organ in each setiger; follicles; isolated oocytes (*)	Paired organ in each setiger; follicles; (*)
Type of acessory cell	Follicular cells (*)	Lack (*)	Follicular cells	Follicular cells	Follicular cells	Follicular cells
Possible source of pre-vitelinic substances	Coelom and follicular cells (*)	Coelom and parenchymal tissue (*)	Blood vessel, gut, follicular cells (*)	Follicular cells and coelom (*)	Gut, follicular cells and close oocytes	Gut, follicular cells and close oocytes

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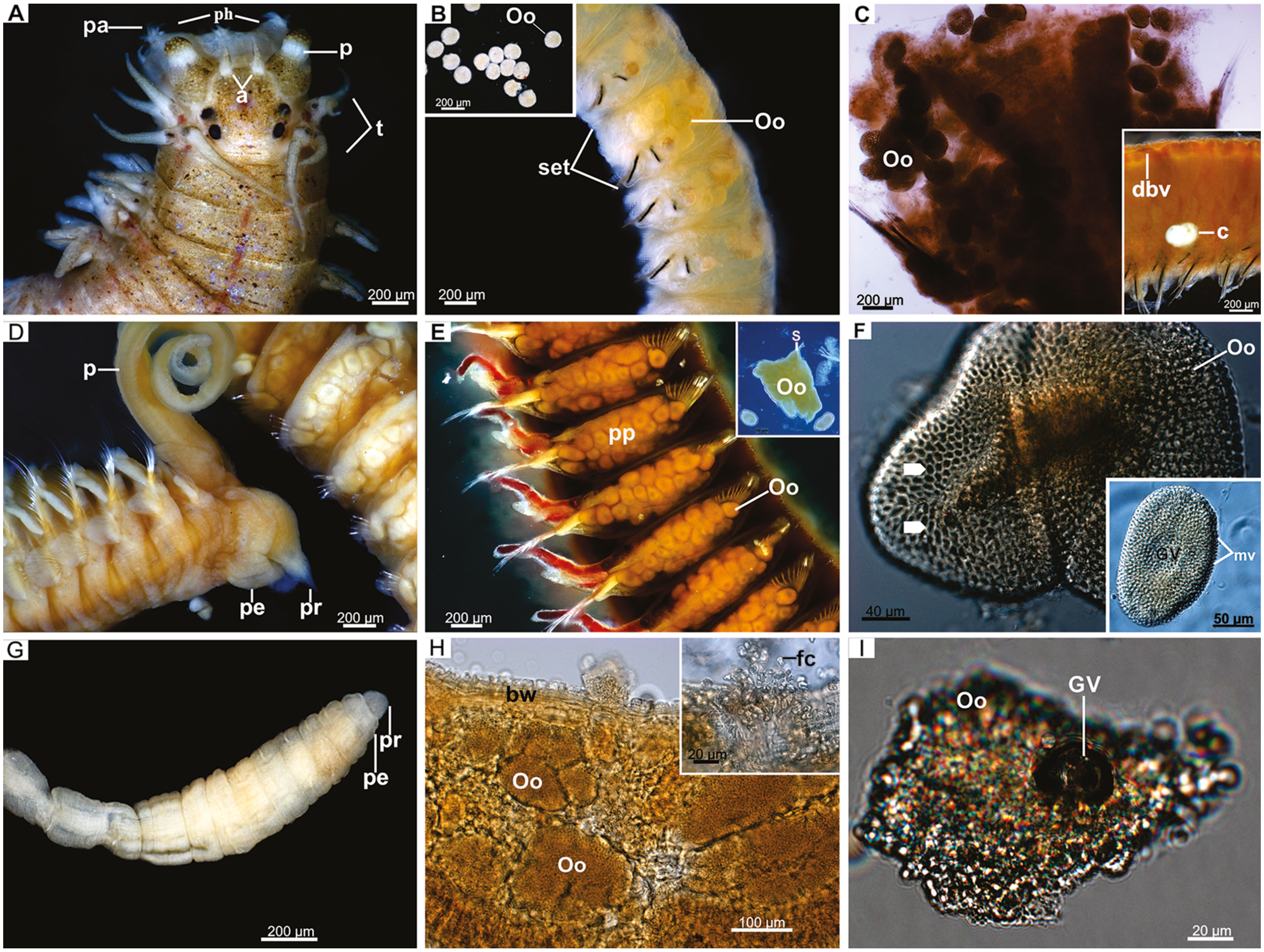

Figure 1. *Laeonereis culveri*, *Scolelepis goodbodyi* and *Capitella biota* external morphology, highlighting the germ cells and its macro organization. **A – C:** *Laeonereis culveri*; **A – Anterior body dorsal** view; **B** – Germ setigers; detail: mature oocytes visible through the tegument; **C** – Release of free oocytes from the coelom by rupture of the body wall; detail: primary and secondary oocytes cluster seen through the female tegument; **D – F:** *S. goodbodyi*: **D** – Anterior body and germ setigers; **E** – Germ setigers in lateral view, showing parapodia full of oocytes; detail: epthelium sheath (sac-like tissue) holding oocytes; **F** – External morphology of oocytes evidencing the organization of honeycomb-like membrane projections; detail: macrovilli; **G – I:** *C. biota*; **G** –Anterior body, dorsal view; **H** – Distribution of follicles in the germ setigers observed through the integument; detail: free follicular cells after body wall disruption; **I** – Detail of a free oocyte released from the follicle. **a** = antennae; **arrowhead** = honeycomb-like structures; **bw** = body wall; **c** = cluster; **dbv** = dorsal blood vessel; **fc** = follicular cell; **GV** = germ vesicle; **mv** = macrovilli; **Oo** = oocyte; **p** = palps; **pa** = pharynx papilae; **pe** = peristomium; **ph** = pharynx**;** **pp** = parapodia; **pr** = prostomium; **set** = setiger; **t** = tentacles.

2.1. Laeonereis culveri

In *L. culveri* no differentiated reproductive system was properly observed and clusters of oocytes in different development stages are free-floating in the coelom (Figure 2A-C). Primary oocytes clusters are free of follicular cell sheath (Figure 2B), which seems to start its connection to germ cells only after the latter become secondary oocytes and initiate vitellogenesis. Primary and secondary oocytes are small and rounded cells whose cytoplasm is homogeneous, free of apparent yolk vesicles and with restricted space due to the size of the nucleus. The nucleus, in turn, is large, also rounded, with condensed peripheral chromatin, occupying most of the intracellular space (Figure 2B, C and E).

Somatic cells that are associated with oocytes are follicular cells that show a heterogeneous cytoplasm and chromatin, suggesting a high synthesis activity, mainly when they are related to oocytes in advanced vitellogenesis (Figure 2D and F). The oocytes observed in this species seems to undergo non-synchronous vitellogenesis, due to distinct characteristics between oocytes inside a single cluster. Oocytes initiating vitellogenesis are characterized by rounded cells, with homogeneous cytoplasm still without yolk vesicles in which the nucleus occupies the center of the cell and presents a loose chromatin, indicating synthesis activity. In other oocytes, morphologically similar to previous ones, few yolk vesicles accumulating at the periphery of the cell are observed. Larger oocytes with a greater yolk accumulation can be seen in the same cluster (Figure 2D-F).

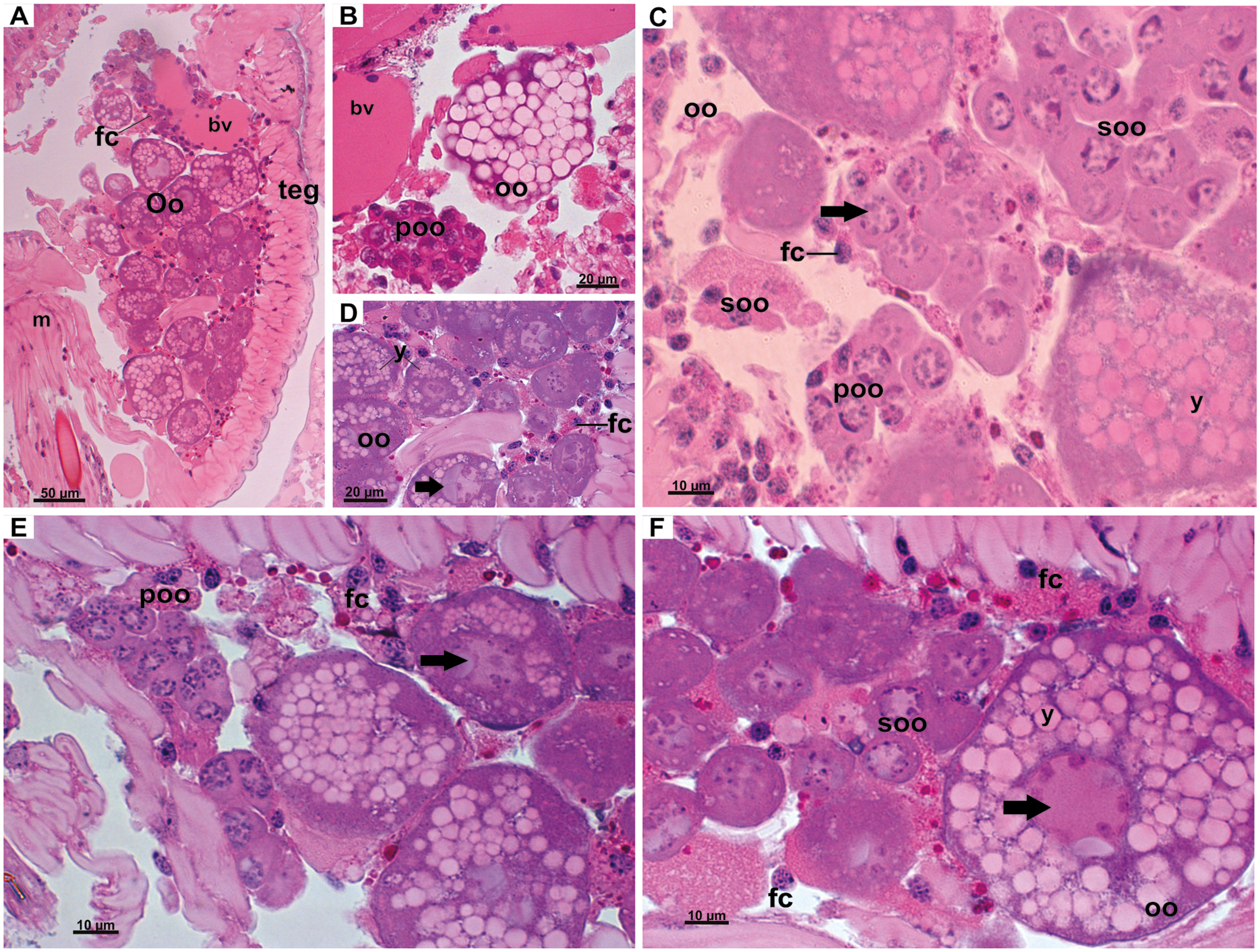

Figure 2. *Laeonereis culveri* histological sections with emphasis on its germ cells and vitellogenesis. **A – D:** Histological sections of parapodia where primary and secondary oocytes clusters are observed, associated to blood vessels and follicular cells (soo); **E – F:** Detail of primary and secondary oocytes clusters and oocytes in vitellogenesis. **Arrow** = germ vesicle (nucleus); **bv** = blood vessel; **fc** = follicular cell; **m** = muscle; **Oo** = oocyte; **poo** = primary oocytes; **soo** = secondary oocytes; **teg** = tegument; **y** = yolk.

In oocytes in which the vitellogenesis is more advanced, the yolk vesicles are becoming larger in a fusion process and in some cases, it is not possible to observe the germinal vesicle (nucleus). In such cases, yolk vesicles occupy the whole of the cytoplasm and the characteristics become striking, even altering the rounded form of the oocyte, and the shell begins to be deposited in the external cellular limit (Figure 2A-C). Furthermore, these oocytes are still observed associated with follicular cells (Figure 2D, E and F).

2.2. Scolelepis goodbodyi

In this species, it is possible to observe an epithelial sheath (peritoneum) that compacts and shelters the oocytes isolating them from the other organs immersed in the female coelomic fluid, restricted to the parapodia (Figure 3A-B). The most immature germ cell stage found here is secondary oocytes, arranged in pairs, without cytoplasmic bridges (as far as can be seen with this technique) and associated to a blood vessel by one of their poles. They are oval/elliptic cells with round nuclei, cytoplasm slightly granular (heterogeneous) but with no evidence of yolk vesicles (Figure 3A).

In this species, oocytes initiating vitellogenesis are cells that have undergone a marked hypertrophy, exhibiting double or more of secondary oocytes' size. They are cells elliptically shaped, whose cytoplasm exhibits signs of yolk granulation. The germinal vesicle retains its round shape, but with little condensed chromatin and a large nucleolus strongly stained by hematoxylin (Figure 3A-B). Continuing the vitellogenesis process, the cells almost double in size and the cytoplasm is already found with a thin yolk granulation easily observed. The formation of membrane specializations, or projections, at the border of the cell create intimate contact with other oocytes, follicular cells and with blood vessels. In the next developmental step, a greater amount of yolk vesicles within the cytoplasm and larger and more developed membrane projections can be observed as oocytes main features (Figure 3B-D).

Among oocytes in final stages of vitellogenesis, a considerable increase in the yolk assimilation and synthesis is observed due to the larger quantity of vesicles, also of larger size, occupying the cytoplasm almost completely, besides a well-developed germinative vesicle with loose chromatin and nucleolus well evident.

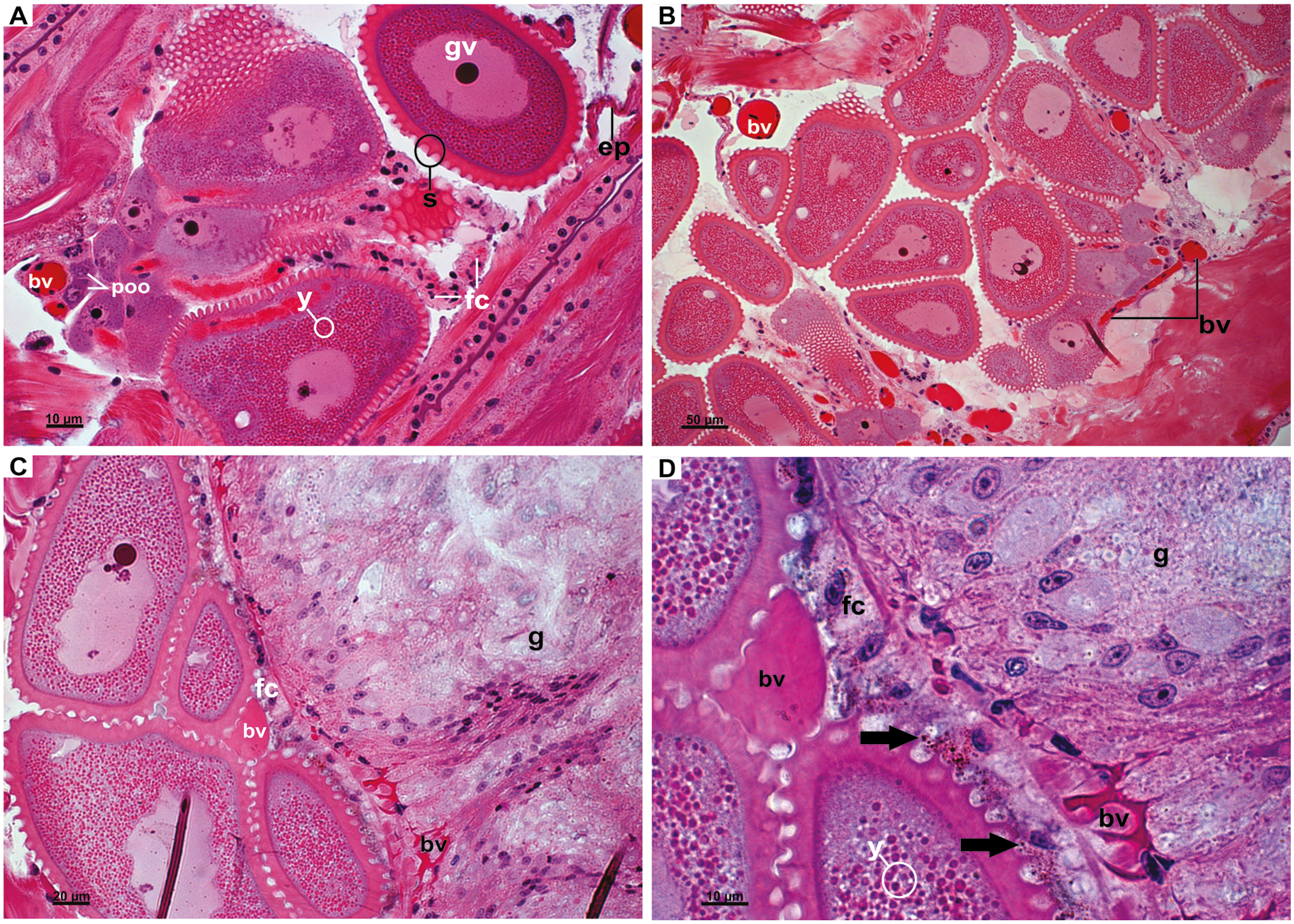

Figure 3. *Scolelepis goodbodyi* histological sections with emphasis on its reproductive system, germ cells and vitellogenesis process. **A:** Parapodia section showing an ovary filled with germ cells from the initial to the advanced stages of development; **B:** Details of blood vessel close to primary oocytes and vitellogenic oocytes; **C:** Detail of blood vessel very close to vitellogenic oocytes and follicular cells, which are in contact with gut wall; **D:** Magnification of previous image, showing a possible material transfer between the gut, blood vessel and follicular cells to oocytes (**arrow**). **bv** = blood vessel; **ep** = epithelium; **fc** = follicular cell; **g** = gut; **gv** = germ vesicle; **poo** = primary oocytes; **s** = shell; **y** = yolk.

At this stage, the shell deposition between the membrane projections is observed, covering the entire layer of villi on the cell surface when complete and tending to lose contact with other cells, including follicle ones (Figure 3A, 3C-D).

The follicular cells found in this species seem to play a role in vitellogenesis, evidenced in the figures 3B and 3D, not only by direct contact with the oocytes, but also by association with blood vessels and by the external wall of the individual's gut. Germ cell support within the epithelial sheath appears to be a function of the follicular cells associated with blind capillaries, where they form peduncles similar to grape clusters, maintaining an interconnected network between follicular cells, gut, blood vessels and oocytes.

2.3. Capitella biota

This species, as others congeneric ones, presents a rudimentary ovary, with a paired and bead necklace organization and its walls are formed by an epithelium anchored in the dorsal peritoneum in its upper portion and longitudinally in the septa of each segment crossing the entire set. The epithelium that connects the organ to the inner face of the body wall and to the septa has cubic cells, while the portion involving each oocyte individually (forming the follicles), exhibits a single layer of pavemented cells (Figure 4A and B).

As previously stated, *C. biota* oocytes are very large cells (250 µm in length) when compared to the body size of the specimen, but in early stages of vitellogenesis, oocytes are tiny cells with few yolk vesicles (which increase in quantity and volume according to development) and a germinal vesicle prominent in the center of the cell (Figure 4A detail). As oocytes progress in vitellogenesis, the cell increases considerably in size and the yolk vesicles also increase in size and quantity while the germinal vesicle is almost not observed (Figure 4B). The largest and most developed germ cells observed here exhibit a rather increased, elongated and elliptical size, taking up half the full length of a setiger of the specimen, with the cytoplasm full of yolk vesicles covering the germinal vesicle (Figure 4C and D).

No cytoplasmic bridges were observed between the oocytes at any stage, but it was possible to notice an intimate contact of the oocytes with the many follicle cells found in the coelom, in addition to a proximity to the gut.

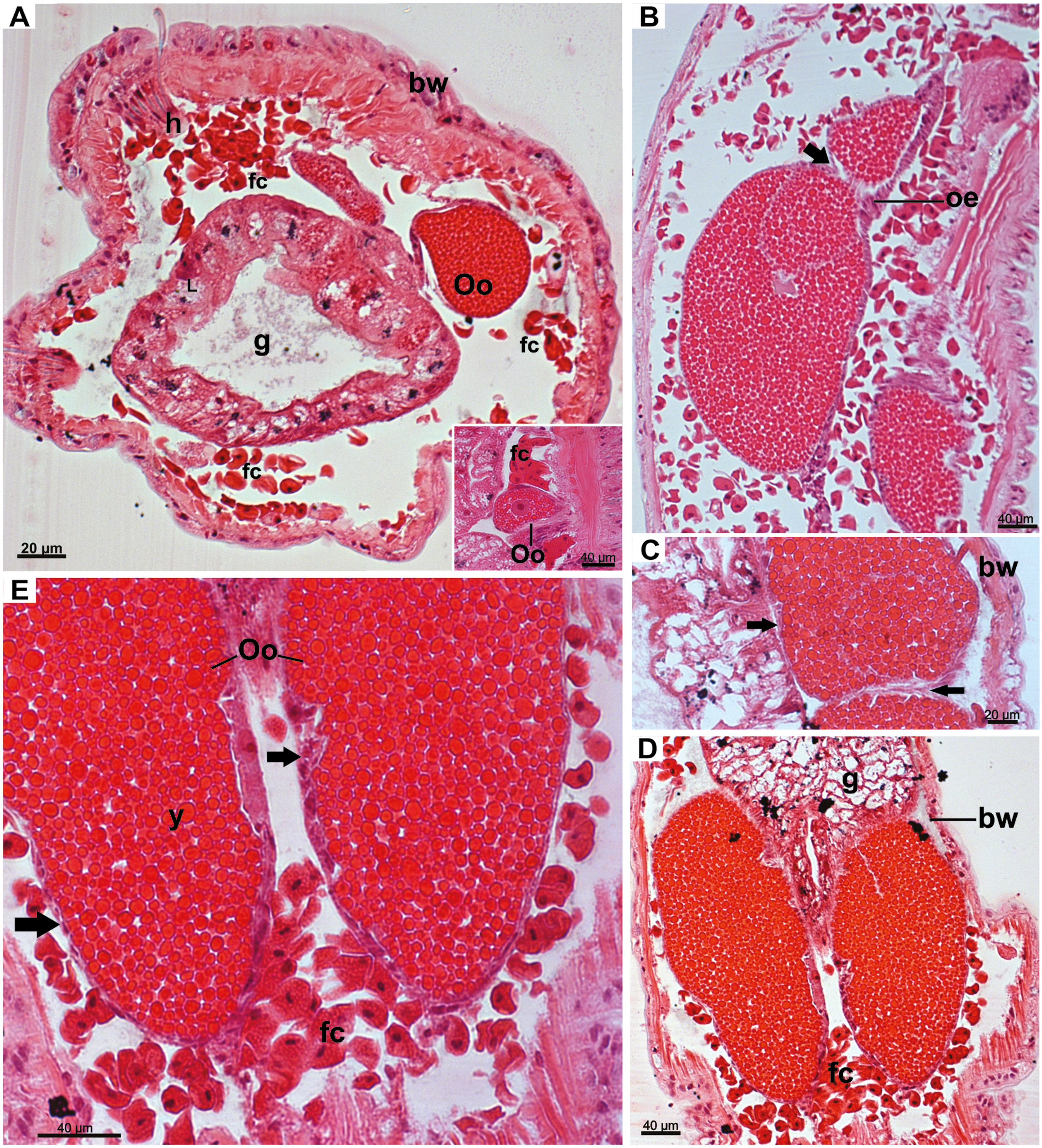

Figure 4. *Capitella biota* histological sections with emphasis on its reproductive system, germ cells and vitellogenesis process. **A:** Transverse section of the germ setiger, where a gut with dilated lumen is observed, surround by several follicular cells and a follicle with an oocyte in vitellogenesis process. Detail: Imature oocyte; **B:** An mature oocyte and the epithelial tissue that involves each germ cell; **C - E:** Details of the ovary epithelium housing each of the oocytes, surrounded externally by follicular cells. **bw** = body wall; **fc =** follicular cells; **g** = gut; **h** = hook; **oe** = ovary epithelium; **Oo** = oocyte; **arrow** = individualized oocytes; **y** = yolk.

These follicular cells are amoeboid-shaped, showing cytoplasm strongly stained by eosin and very heterogeneous, with a small and condensed nucleus, found in clusters associated with the ovary itself and also with the gut (Figure 4A-D).

3. *Molecular analysis*

For the three species studied here, the ML trees clearly discriminated them from geographic distinct populations (*Laeonereis culveri* – Figure 5A) or from congeneric species (*Scolelepis goodbodyi* – Figure 5B and *Capitella biota* – Figure 5C). Mean pairwise COI distances between *S. goodbodyi* and the other congeneric species ranged between 15 and 23%, between *C. biota* and the other congeneric species ranged between 18 and 21%, while for *L. culveri*, the pairwise distance between the populations from Brazil and North America was 16.78%.

Discussion

When comparing the species in this study regarding the shape and size of their oocytes, there are marked variations that reflect relevant ecological and/or reproductive aspects. In *L. culveri* the largest oocytes reach little more than 150 µm in diameter at the end of vitellogenesis and have a rounded/spherical shape, while the oocytes of *S. goodbodyi* and *C. biota* are bigger than 200 µm in length and have an elliptical shape when packed in the ovaries; also, in *C. biota*, the oocytes present a less definite shape when outside the ovary. These characteristics, based on Adiyodi & Adiyodi (1983), separate *L. culveri* from the other two species (as expected) due to the presence of large numbers of oocytes of smaller sizes, classifying it as a species of discrete iteroparity, whereas *S. goodbodyi* and *C. biota* show a different reproductive strategy, i.e., semi-continuous reproduction, in which individuals produce a smaller number of eggs, but with a larger size. *C. biota* has the smallest number of oocytes compared to the other two species, with ovarian follicles and individualized oocytes. Thus, with respect to the morphology and development of the female reproductive system, these three species are very distinct and well defined in their respective families.

Considering the studies carried out by several authors, including the present one, it is believed that the oogenesis in polychaetes occurs in two ways (Eckelbarger 2005), namely extraovarian (**a**) and intraovarian (**b**): **a)** final differentiated oogonia or primary oocytes detach from their proliferative tissue and reach the coelomic cavity, where they conclude development; and **b)** in which oocytes develop completely within an ovary, usually associated with follicular cells (Wilson 1991, Eckelbarger 1994, 2001, 2005) . In this study, the authors propose a subdivision of the two types described by Eckelbarger (2005), both of which display two subtypes:

a) extraovarian type I – the oocyte is released from the proliferative tissue as primary oocytes (pre-vitellogenic), individualized (solitary) within female's coelomic cavity, where full development occurs.

b) extraovarian type II – clusters of primary (pre-vitellogenic) oocytes, surrounded by a follicular cell sheath and/or a binder matrix, are released into the coelomic cavity for the entire process to occur.

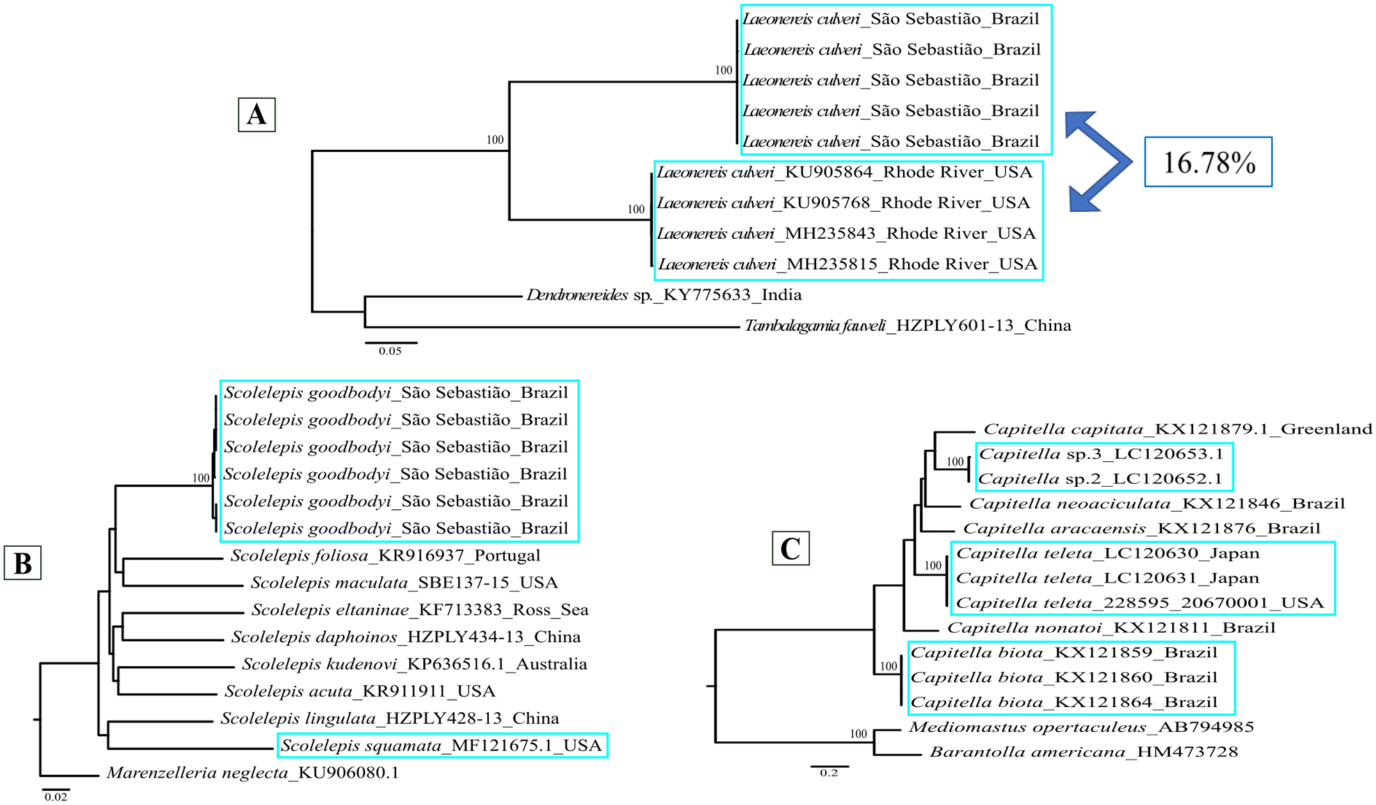

Figure 5. Phylogenetic trees of the species herein studied. Numbers by the nodes indicate respective maximum likelihood bootstrap values; values below 90 not shown. **A:** ML phylogenetic COI tree of *Laeonereis culveri*. The species *Dendronereides* sp. and *Tambalagamia fauveli* were used as outgroup. **B:** ML phylogenetic COI tree of *Scolelepsis goodbodyi*. The species *Marenzelleria neglecta* was used as outgroup. **C:** ML phylogenetic COI tree of *Capitella biota*. The species *Mediomastus opertaculeus and Barantolla americana* were used as outgroup. The COI sequence for *Capitella teleta* from USA is deposited under ID 228595, on the Genome Portal of the Department of Energy Joint Genome Institute (ESC-2004).

c) intraovarian type I – oogonias housed inside an epithelial sheath/sack proliferate and primary oocytes develop until the late phase of vitellogenesis, released into the coelomic cavity afterwards, ending this process and subsequent oviposition/fertilization.

d) intraovarian type II – oocytes packaged in epithelial sheath/sack, fully develop in their interior until the ovulation period.

This complementary data regarding oogenesis paths in polychaetes brings to light additional characters that can be used to differentiate other taxonomic levels, such as genus and species. Each species-case treated herein is discussed below, encompassing this and others characters observed, aiming to show how the FRS histology could be used as a tool to discriminate species.

1. *The* Laeonereis culveri *case: cryptic or cosmopolitan?*

In *L. culveri* from São Sebastião, Brazil, there is no true ovary. In this species, the germinative tissue (not observed herein) is distributed in pairs in the setigers and associated to blood vessels, and its observation depends on the technique used and life cycle stage (Eckelbarger 2005). The germ line cells originate from this tissue and the follicular ones from the peritoneum. In this way, ovulation in *L. culveri* occurs prior to the onset of vitellogenesis, consisting of a large number of primary oocytes being produced and released in clusters within the adult coelom, where the maturation occurs. The absorption of pre-vitelline material probably occurs in the coelomic fluid; firstly, directly from the fluid, when primary oocytes are clustered with no follicular cell support; posteriorly, secondary oocytes receive follicle cells support and the absorption probably occurs through these cells. In this way, the oogenesis in *L. culveri* from Brazil can be classified as extraovarian type II.

Klesch (1970) studied the same species collected in Texas, USA, and observed that the oogenesis begins in the peritoneal germinative tissue, and oogonia clusters, agglutinated by a basophilic matrix, are released from this tissue. Afterwards, already as primary oocytes, the germ cells release from each other and, individually floating in the coelom, go through the whole vitellogenesis process. All germ cells observed and described by Klesch (1970) are spherical, and the mature oocyte exhibit a thin shell in its external surface. Furthermore, the author did not observe true follicular cells associated and/or attached to germ cells, suggesting that peritoneum cells should play this role. To illustrate the comparison between Klesch´s findings and ours, we provided herein a schematic illustration side-by-side to clearly shown the divergent histological features that take us to our inferences (Figure 6).

In the same way, Florêncio (1999) also observed oocytes floating in the coelom at the beginning of vitellogenesis and in the final phase of this process in individuals identified as *Laeonereis acuta* (Treadwell 1923), collected on the beach of Enseada dos Corais, Pernambuco, Northeast Brazil. This congeneric species is presently considered to be a junior synonym of *L. culveri* (Oliveira 2009; Read & Fauchald 2018), so it can be assumed that it is a different population of *L. culveri* of the Brazilian coast. In the same study, gametes in the proliferative phase (oogonia) were observed in a single individual as cell-agglomerates without the presence of a cellular sheath and the author never mentioned follicular cells associated.

Thus, *L. culveri* specimens from Texas and *L. culveri* specimens from Pernambuco (*L. acuta*) present an extraovarian oogenesis type I, with no follicular cell associated and the yolk precursors probably absorbed directly from coelom. Klesch (1970) suggest also a participation of parenchymal cells in this role. On the other hand, *L. culveri* specimens from São Sebastião (this study) present an extraovarian oogenesis type II with an association of follicular cells, which houses oocytes in clusters until later phases of vitellogenesis. Furthermore, the oocyte morphology and the shell deposition in mature oocytes seem to be very important characters which can operate as reproductive barriers between distinct correlated species. These marked differences among individuals from geographically distinct populations of *L. culveri* suggest the existence of at least two lineages, probably more, on the Atlantic coast of the Americas, reinforcing the indication that it is a case of cryptic species.

Herein, we are considering *L. culveri* from Texas as a different population primarily to fit in our purpose, but also because Klesch (1970) identified these specimens as *L. culveri*. As our goal is to demonstrate the histology of FRS as a good method to complement cryptic species complexes elucidation, it seems to be scientifically valid comparing our results to those found by Klesch (1970), as well as to make use of *L. culveri* barcodes from other regions from USA. In this sense, other authors, such as Oliveira (2009) and Oliveira et al. (2010), performed morphological analysis attempting to diagnose characters variation within several different populations of *Laeonereis* and concluded that *L. culveri* is a truly cosmopolitan species, which shows morphological variability not related to geographical occurrence, rather to environment contamination and fixation techniques. Those findings corroborate Pettibone (1971), in which four species were synonymized with *L. culveri*, also concluding that *L. culveri* is a truly cosmopolitan species, but limited to the North and South Atlantic coast of the Americas (Jesús-Flores et al. 2016).

Our findings regarding the FRS histology and DNA barcode strongly support the hypothesis that *L. culveri* represents a species complex and the histological features of the reproductive structures differ among the specimens of *L. culveri* among the populations herein compared (Klesch 1970, Florêncio 1999, this study). Such differences may also indicate the existence of reproductive barriers between individuals of different populations, therefore possibly revealing a process of speciation.

The COI sequence data obtained for *L. culveri* corroborate the inferences based on the histological observations. The specimens from São Sebastião, Brazil show an intraspecific distance of 16.8% from specimens collected in the Rhode River, USA, also identified as *L. culveri*. This genetic distance is considerably higher than the most frequently observed values for maximum intraspecific distances (about 2 to 3% only) in comprehensive analyses of various invertebrate taxa (Ratnasingham & Hebert 2013), including polychaete fauna (Lobo et al. 2016) which leads to the strong indication that these are two distinct species, and their reproductive features are relevant. Analyses of additional specimens from other localities, particularly from Texas and along the Brazilian coast, are required to gain a comprehensive picture on the taxonomic status of this species, mainly searching for morphological characters that allow species differentiation, as well as for molecular data for species delimitation.

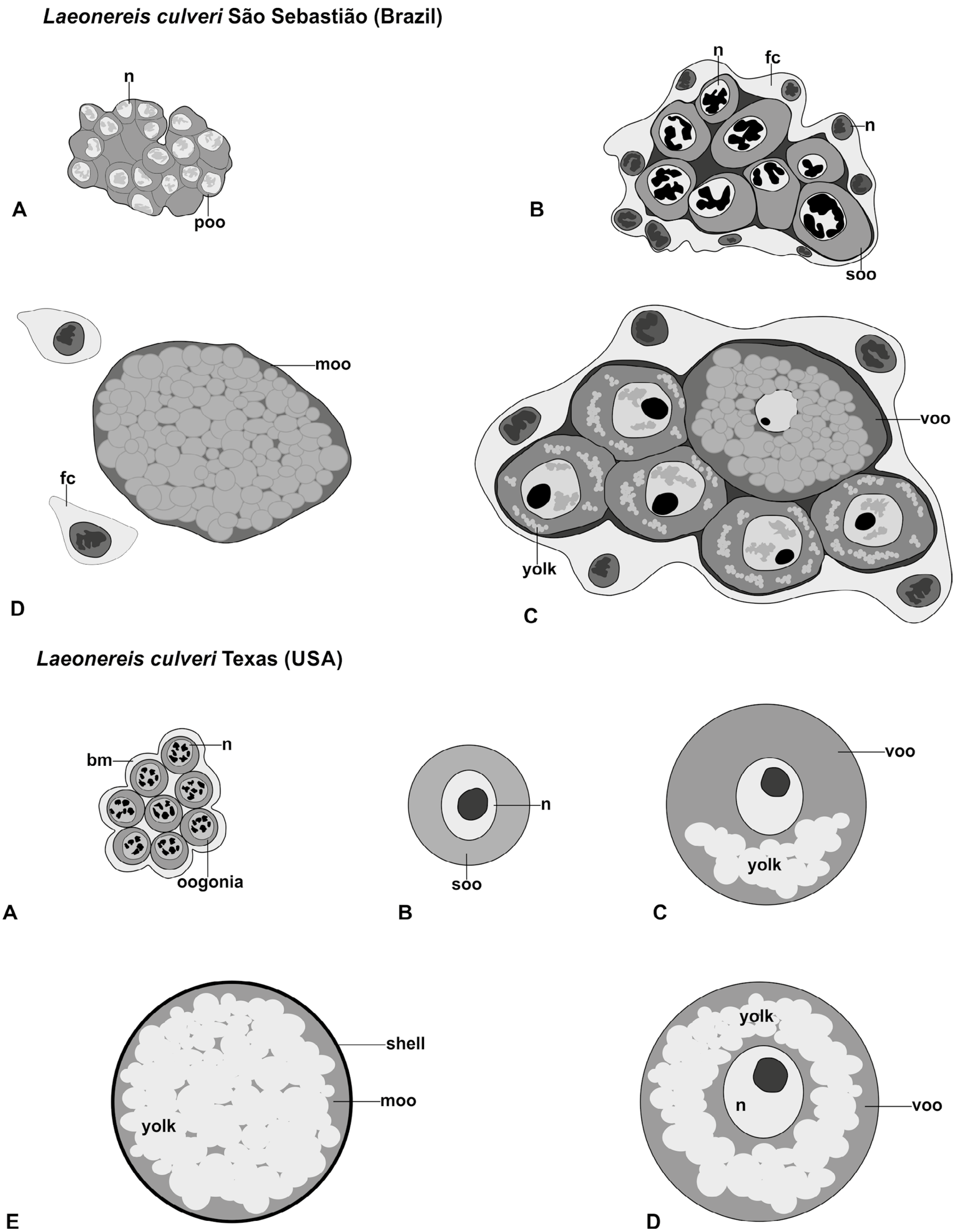

Figure 6. Schematic illustration comparing the oogenesis process in two distinct populations of *Laenereis culveri*. ***Laeonereis culveri* from São Sebastião (Brazil):** **A** – Primary oocytes cluster; **B** – Secondary oocytes clusters wrapped by a follicular cell sheath; **C** – Vitellogenic oocytes inside the follicular cell sheath; **D** – Mature oocyte released from the sheath. ***Laeonereis culveri* from Texas (USA):** **A** – Cluster of oogonia; **B** – Secondary oocyte; **C** and **D** – Vitellogenic oocytes; **E** – Mature oocyte. **bm** = basophilic matrix; **fc** = follicular cell; **moo** = mature oocyte; **n** = nucleus; **poo** = primary oocyte; **soo** = secondary oocytes; **voo** = vitellogenic oocyte.

2. *The genus* Scolelepis: *a well-defined taxa*

The follicular cells of *S. goodbodyi* exhibit a peculiar organization; they are distributed externally along blind-capillaries until reaching the oocytes. These oocytes produce numerous macrovilli (membrane specializations), which allow them to connect with the follicular cells and other tissues, creating a network between blood vessels, follicular cells and oocytes. This complex configuration shows a high-level specialization of the cellular and subcellular structures related to vitellogenesis process, not yet described in the literature for polychaete species.

Richards (1970), with the study of the reproductive biology of *Scolelepis squamata* (Müller, 1806) collected in Barbados (Caribbean), reported similar morphological, histological and physiological aspects to those observed here for *S. goodbodyi*, such as lateral ovary (associated to blood vessels) formed by peritoneal epithelium, as well as oval-shaped oocytes with macrovilli external to the shell zone. However, the author pointed out the occurrence of loose oocytes in the coelomic cavity of the specimens in the final phase of vitellogenesis (*i.e.*, intraovarian type I) whereas an intraovarian type II oogenesis was described here for *S. goodbodyi*. For *S. squamata* there is no report of a network between the oocyte macrovilli and the follicular cells, or even with the intestine and blood vessels (Richards 1970), being a striking feature observed here for *S. goodbodyi*. In this sense, it is supposed that in *S. squamata* the interconnected network that optimizes the vitellogenesis does not occur in any way and the macrovilli function for this species is only to increase the absorption area. This characteristic should be considered as a relevant interspecific variation for species (re)description and differentiation within the genus *Scolelepis*.

Blake & Arnofsky (1999) considered the anatomical position of the ovaries and the ultrastructure of the ovary envelope as the most relevant characters of the female reproductive system for phylogenetic analysis and species differentiation in Spionidae. According to the comparison between *S. squamata* and *S. goodbodyi* regarding the differences of the oogenesis types (intraovarian type I and type II, respectively) and the macrovilli network, these characters should be used for further phylogenetic and/or taxonomic analysis (Richards 1970, Blake & Arnofsky 1999, Eckelbarger 2001). The genetic data obtained show *S. goodbodyi* as a well-defined distinct group, which has a divergence of 19.66% from *S. squamata* from the USA, reinforcing that histological features could be relevant for integrative studies regarding Spionidae.

3. *The genus* Capitella: *messing up minds since the 17th century*

According to Blake (2009), studies carried out by several authors in the last 30-40 years about classification, occurrence and distribution, ecology and morphology of the supposedly cosmopolitan species *Capitella capitata* (Fabricius 1780) have revealed, in fact, a complex of species with a very similar morphology (internal and external). Grassle & Grassle (1976) and Eckelbarger & Grassle (1982, 1983) demonstrated the existence of approximately eight to twelve distinct species occurring on the North American coast. In these studies, the authors recognized and described six sibling species (named *Capitella* sp. I, Ia, II, IIa, III and IIIa) through life history traits and reproductive features, including ovary morphology and oogenesis.

Blake et al. (2009) described and named one of these sibling species, the *Capitella* sp. I, as *Capitella teleta* Blake, Grassle & Eckelbarger 2009, using morphological characters, life history features, and the COI gene sequence. Posteriorly, Tomioka et al. (2016), working with morphological identification and DNA barcode (COI) of the same species, confirmed that *C. teleta* has a real cosmopolitan distribution, as previously supposed, certain phenotypic plasticity and intraspecific variation of a couple of morphological characters. Another sibling species already identified is *Capitella* sp. III, as *Capitella jonesi* (Hartman, 1959) by Eckelbarger & Grassle (1982); however, the other sibling species remain unidentified.

Capitella teleta and *C. jonesi* exhibit a very similar morphology and histology of the reproductive system, except in the number and average size of the oocytes and their yolk composition. However, those characters may vary according to environmental conditions and/or ecological features, such as food-type availability, contaminants, sex ratio and local abundance, demanding an integrative approach, including DNA barcoding, to separate them into distinct species (Eckelbarger 1994, 2001, 2005, Blake & Arnofsky 1999).

Capitella biota was recently described for the Brazilian coast after an extensive review of specimens previously identified as *C. capitata*, which is known to be restricted to its type locality (Greenland, Arctic Circle) (Blake 2009, Tomioka et al. 2016, Silva et al. 2017). The female reproductive system of this species exhibits characteristics similar to *C. teleta* and *C. jonesi* corroborating the hypothesis that it is a highly complex morpho-physiological model within polychaetes (Eckelbarger & Grassle 1982, 1983, Blake et al. 2009, Silva et al. 2017).

The paired ovaries in the reproductive chetigers with sac-like follicles, delimited by a wall of flattened epithelial cells, anchored laterally and dorsally on the body walls and on the septum, respectively, are common characters for the genus *Capitella* and have also been observed in *C. biota, C. teleta* and *C. jonesi*. Nevertheless, it was possible to highlight an important difference between *C. biota* and the other species studied: the ovarian wall isolates the oocytes, housed inside the follicles, since pre-vitellinic stages and being separated from the follicular cells. Eckelbarger & Grassle (1982, 1983) described an antagonistic situation for *C. teleta* (*Capitella* sp. I) and *C. jonesi* (*Capitela* sp. III). In both, the oocytes are surrounded by follicular cells that fill the spaces between the oocytes at different stages of vitellogenesis, and this whole set of germinal and follicular cells are, in turn, packaged in a sheath of epithelial cells (ovarian wall). It is then believed that the shape, distribution and tissue organization of follicular cells, as well as the organization of follicles could represent an interspecific variation that allows the separation of species of the genus *Capitella*.

The COI sequence data presented here for *Capitella* bring relevant findings for the taxonomic issues of this genus:

a) The tree exhibits *C. biota* as a very distinct group from the other species with a genetic distance between 18 and 20% from *C. teleta* and *C. jonesi*;

b) The *Capitella* sp. II (2) and sp. III (3) are the same species, probably *C. jonesi*, justifying why they have almost identical reproductive system and external morphology;

c) *Capitella teleta* from the USA has a genetic distance of almost 18% from *Capitella* sp. II and III (*C. jonesi*).

The differences observed between the ovaries of *C. biota* and the other two congeneric species corroborates that this is indeed a distinct one within the complex, as demonstrated by Silva et al. (2017). In addition, the genetic data shows how intricate is the group, housing at the same time cryptic and cosmopolitan species. Thus, our comparative analysis demonstrates the value of histological features from the FRS to help solving misleading classification and species descriptions within the *Capitella* complex.

4. *The outcome so far*

Given the great variety of characteristics of polychaete female reproductive system, a phylogenetic study based on these characters seems to be unpractical and time-consuming, mainly in a scenario where molecular tools are taking up space in a fast way. Nevertheless, it is likely that such information, when generated through extensive sampling and application of varied techniques, may bring a multi-tool approach to separate correlated species, such as true cryptic and cosmopolitan ones.

The microhabitat of small benthic species, such as the interstitial (meiofauna) and the infauna, has a great influence on its phenotypic modulation, for example, related to the reproductive behavior (cohort, parental care) and such as the morphology of the reproductive system. In this sense, there are indications that this wide variety of reproductive modes and gametogenesis/vitellogenesis types in polychaetes, in some cases, are associated with the environment in which these individuals occur, as well as the overlap of ecological niches (Eckelbarger 2001, Rouse & Pleijel 2001, Katz & Rouse 2013).

From time to time, researchers trying to understand genetic patterns that indicate the occurrence of highly divergent lineages within same species and associated geographic distributions, are appealed to the classical morphological techniques, as well as histological ones, to obtain satisfactory answers (Blake & Arnosfsky 1999, Sato & Nakashima 2003, Lobo et al. 2016;). Lack of diagnostic morphological characters constitutes a major hindrance for the description and widespread acceptance and recognition of the numerous cryptic species of polychaetes and other invertebrates that have been detected over the last years. The "*Laeonereis* complex" is a good example of this. Our study indicates that the histology of ovary and oocytes could constitute a tool that can be added to molecular methodologies, thereby greatly assisting the description, re-description, and systematic analysis of cryptic and pseudo-cryptic species of these annelids, and eventually other invertebrate species.

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Author Contributions

Bruno R. Sampieri: Substantial contribution on the work conception and design, as well as with the data collection and analysis.

Tatiana M. Steiner: Contributed on data acquisition and text writing.

Camila Fernanda da Silva: Contributed on data acquisition and text writing.

Priscila C. Baroni: Contributed on data acquisition and text writing.

Marcos A. L. Teixeira: Contributed with data analysis and interpretation as well as with text writing.

Pedro E. Vieira: Contributed with data analysis and interpretation as well as with text writing.

Antonia C. Z. Amaral: Contributed substantially to its development.

Filipe O. Costa: Contributed substantially to its development.

Conflicts of Interest

The authors have no conflict of interest to declare.

Data availability

All DNA sequences used in the present study and obtained by the authors through sampling were deposited and published in Genbank and in the Barcode of Life Database (BOLD).

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The role of spatial and environmental variables in shaping aquatic insect assemblages in two protected areas in the transition area between Cerrado and Amazônia

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Abstract: The distribution of aquatic insects of the orders Ephemeroptera, Plecoptera, and Trichoptera (EPT) can be influenced by factors such as water quality, habitat integrity and biogeography. The present study evaluated the structure of EPT assemblages in streams in the Cerrado, a global biodiversity hotspot. Samples were collected from 20 streams in two protected areas: Parque Estadual do Mirador (10 streams) and Parque Nacional da Chapada das Mesas (10 streams). A total of 1987 specimens were collected, representing 46 taxa of EPT. The two study areas did not differ significantly in taxonomic richness of EPT genera (t = -1.119, p = 0.279) and abundance of individuals (t = 0.268, p = 0.791) but did differ in genus composition (Pseudo-F = 2.088, R2 = 0.103, p = 0.015) and environmental variables (Pseudo-F = 2,282, R^2 = 0.112, p = 0.014). None of the tested environmental variables were correlated with the community but a spatial filter captured an effect of the spatial distribution of streams. The region of the study is located in MATOPIBA, which is the last agricultural frontier of the Cerrado. Therefore, it is important that there is police and monitoring so that the "Parque Estadual do Mirador" and the "Parque Nacional da Chapada das Mesas" continue to play their role in conserving biodiversity in the future.
Keywords: *Biogeography, Conservation units, EPT, Stream ecology.*

O papel das variáveis espaciais e ambientais na formação de assembleias insetos aquáticos em duas áreas protegidas na área de transição entre Cerrado e Amazônia

Resumo: A distribuição de insetos aquáticos das ordens Ephemeroptera, Plecoptera e Trichoptera (EPT) pode ser influenciada por fatores como qualidade da água, integridade do habitat e biogeografia. O presente estudo avaliou a estrutura das assembleias do EPT em riachos do Cerrado, um *hotspot* de biodiversidade global. Foram coletadas amostras em 20 riachos em duas áreas protegidas: Parque Estadual do Mirador (10 riachos) e Parque Nacional da Chapada das Mesas (10 riachos). Um total de 1987 espécimes foram coletados, representando 46 táxons de EPT. As duas áreas de estudo não diferiram significativamente na riqueza taxonômica dos gêneros EPT (t= -1,119; p= 0,279) e abundância de indivíduos (t= 0,268; p= 0,791), mas diferiram na composição do gênero (Pseudo-F= 2,088, R^2= 0,103; p= 0,015) e variáveis ambientais (Pseudo-F= 2,282; R^2= 0,112; p= 0,014). Nenhuma das variáveis ambientais testadas foi correlacionada com a comunidade, mas um filtro espacial capturou um efeito da distribuição espacial dos riachos. A região do estudo está localizada em MATOPIBA, que é a última fronteira agrícola do Cerrado. Portanto, é importante que exista fiscalização e monitoramento para que o "Parque Estadual do Mirador" e o "Parque Nacional da Chapada das Mesas" continuem desempenhando seu papel na conservação da biodiversidade no futuro.
Palavras-chave: *Biogeografia, Ecologia de riachos, Unidades de Conservação, EPT.*

Introduction

The spatial distribution of species can be influenced by environmental, spatial and biogeographic characteristics (Leibold et al. 2004, 2010; Crisp et al. 2011), the combination of which is important for understanding how communities are distributed across a landscape and the patterns and mechanisms involved (Presley et al. 2010). Areas of transition, such as between forest-dominated regions (Amazonia) and savannas (Cerrado), are known as ecological tension zones (Marimon et al. 2014; Marques et al. 2019). Species composition tends to vary in such tension zones because of the paradoxical influence of two biogeographically distinct ecosystems located in juxtaposition (Marimon et al. 2014). So, in this transition areas it is expected that biogeographic characteristics play an important role in explaining the distribution of the species (Juen et al. 2017).

Distribution patterns of local communities of aquatic insects of the orders Ephemeroptera, Plecoptera, and Trichoptera (hereafter EPT) are influenced by environmental, spatial, and biogeographic characteristics, however the relative role of these predictors vary depending on the systems. For example, habitat structure in-stream, water quality and habitat integrity (Rosenberg & Resh 1993; Thorp et al. 2006; Allan & Castillo, 2007). The spatial configuration of communities reflects interactions between biotic characteristics, such as dispersal capacity, and abiotic factors, including the presence of geographic barriers, which act at larger spatial scales (Chase & Myers 2011; Dale & Fortin 2014; Vellend et al. 2014). In addition to the types of predictors and spatial scale, species richness and composition, as well as the abundance of individuals, stand out as good descriptive metrics of communities (Juen et al. 2014). This is because species richness can serve as a proxy for alpha diversity (local scale) and species composition as a proxy for beta diversity (differences in species composition among sites) (Jost 2007), while abundance can reflect variation in conditions and resources that influence population size (Tokeshi 1993) and all aspects of the aforementioned diversity are more dynamic in the transition zones between biomes (Ferro & Morrone 2014).

Some transition areas in the Neotropical region are experiencing strong and rapid land-use changes, with negative consequences for biodiversity (Gardner et al. 2013). It is the case of the deforestation arc between Amazonia and Cerrado (Brando et. al 2013), particularly in the MATOPIBA region (Spera et al. 2016). It is critical to understand the distributional patterns of biodiversity in these transition areas to conserve them (Marques et al. 2019). Considering that protected areas are a cornerstone of biodiversity conservation, it is urgent to know the patterns of aquatic biodiversity in protected areas located in tropical transition ecosystems.

In this context, the present study evaluated the structure of EPT communities in two protected areas in the state of Maranhão, Northeast Brazil, an transition area between Amazonian and Cerrado systems: Parque Estadual do Mirador (PEM) and Parque Nacional da Chapa das Mesas (PNCM), with the former being located further from Amazonia and the latter, which is located more centrally in the Cerrado. Since the studied streams are located within strictly protected areas, and thus subjected to minimal anthropogenic impact, it is assumed that the principal mechanisms determining differences in the composition of genera will be geographic distance among streams (spatial autocorrelation) and biogeography, because regions closer to the Amazon will vary in composition due to the more intense dynamics of transition regions. Thus, the present study aimed to test the hypothesis that the two protected areas would not differ in the richness of genera and abundance of individuals but would differ in the composition of genera.

Material and methods

1. *Study area*

The present study focused on two protected areas in the Brazilian state of Maranhão: Parque Estadual do Mirador (PEM) and Parque Nacional da Chapada das Mesas (PNCM) (Fig. 1). Parque Estadual do Mirador was created by Maranhão State decree number 641, of June 20 1980, with an initial area of 450,838 ha, and was subsequently expanded by Law nº. 8.958 of May 8 2009, to a total area of 766,781.00 ha (Maranhão 2009). It includes parts of the municipalities of Mirador, Grajaú, and São Raimundo das Mangabeiras, and is located between the headwaters of the Itapecuru and Alpercartas rivers (06º26’01” S, 44º53’58” W). The predominant vegetation of PEM is Cerrado *sensu lato* a (Conceição & Castro 2009), while the main vegetation types of the studied region are areas of cerradão with the presence of buritizais (growths of Buriti palm trees). The climate is sub-humid to humid (*Aw* in the Köppen classification system), with annual precipitation ranging 1,200 – 1,400 mm, mean maximum temperatures ranging 31.4ºC – 33.0ºC, and mean minimum temperatures ranging 19.5ºC – 21.0ºC (ICMBIO 2016).

Parque Nacional da Chapada das Mesas (7º02’39.6” S, 047º26’28.0” W) was created on December 12, 2005, with a total area of approximately 160,000 ha. It is in the municipalities of Estreito, Carolina, and Riachão, within an area dominated by sandstone formations, which vary in altitude from 250 m in valleys to approximately 524 m on plateaus (MMA 2007). The park has more than 400 springs in its interior that supply the city of Carolina and three important hydrographic basins (Parnaíba, Tocantins and São Francisco). Its main watercourses are the Farinha River in the north and the Itapecuru River in the south (MMA 2016).

The climate of PNCM is tropical humid (*Aw* in the Köppen classification system) with high temperatures throughout the year. There are two well-defined seasons: a dry season from May to October, and a rainy season from November to April. The mean annual temperature is 26.1°C, with minimum temperatures ranging from 25.2°C in January to 27.8°C in September, and maximum temperatures of approximately 36°C in July and August. Annual precipitation ranges 1,250 – 1,500 mm (MMA 2007).

It should be noted that both PEM and PCNM are located in the last agricultural frontier of the Brazilian Cerrado known as MATOPIBA, an acronym derived from the abbreviations of the states of Maranhão, Tocantins, Piaui and Bahia (Spera et al. 2016). To determine anthropic impacts, ICMBio recommends monitoring the fauna and environmental conditions of streams within protected areas (Brasil et al. 2020).

2. *Collection of immature insects*

Specimens of immature insects were collected in May 2018. A total of 10 streams were sampled in each protected area by selecting a 50-m stretch and dividing it into five 10-m segments. All the different microhabitats in which aquatic insects are typically found, including leaf litter, rocks, trunks, macrophytes, and roots, within each segment were examined systematically (adapted from Couceiro et al. 2012).

Insects were collected using an aquatic entomological hand-net (known in Brazil as a “rapiché”) with a 1-mm mesh and manually using a pair of tweezers. Each 10-m segment of each stream was sampled for 15 min. Sampling was replicated three times in each segment.

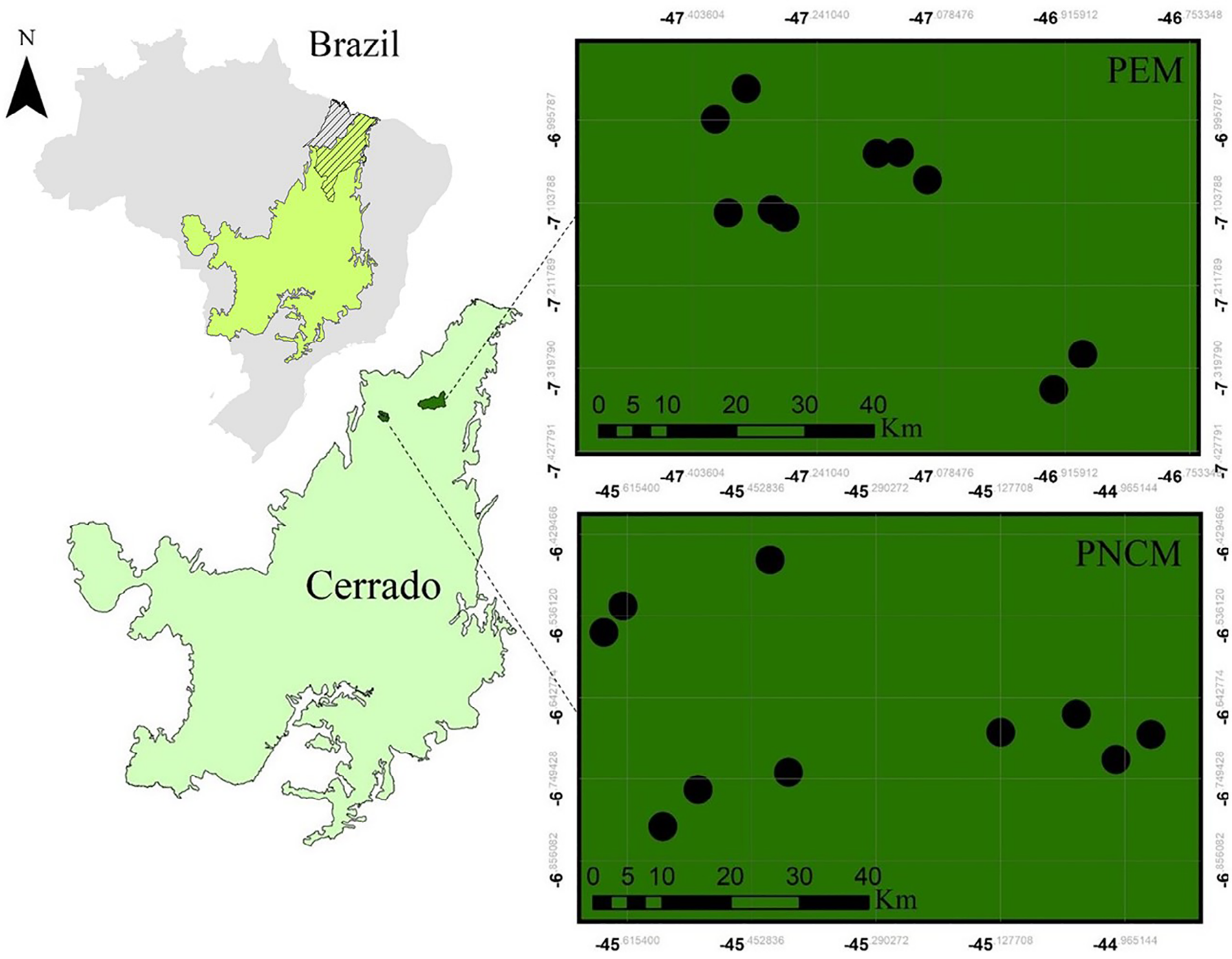

Figure 1. Distribution of the samples (black circles) of aquatic insects in two protected areas of the Brazilian Cerrado, the Parque Nacional da Chapada das Mesas (PNCM) and Parque Estadual do Mirador (PEM).

An initial field screening was undertaken to separate nymphs from the substrate (predominantly leaf, macrophyte, stone and root) with the aid of trays and tweezers. Insect specimens and substrate samples were stored in 80% alcohol in plastic bags and taken to the Laboratório de Entomologia Aquática (LEAq) of the Centro de Estudos Superiores da Universidade Estadual do Maranhão (CESC-UEMA) for further sorting and identification. In the laboratory, substrate samples were washed with water using an entomological sieve, and the nymph specimens separated using a white tray, tweezers and a Stemi DV4 stereomicroscope (Zeiss).

After sorting, the specimens were identified to genus using identification keys for EPT genera, including Costa et al. (2006), Mugnai et al. (2010), Dominguez et al. (2006), Falcão et al. (2011), Salles et al. (2014), Hamada & Silva (2014), and Pes et al. (2014). The specimens were then deposited in the LEAq collection at CESC-UEMA in Caxias, Maranhão, Brazil.

3. *Environmental predictors*

Nine environmental predictors were measured for each stream: width (1), depth (2), environmental integrity (3), pH (4), electrical conductivity (5), temperature (6), dissolved oxygen (7), stream discharge (8) and current velocity (9). Temperature, pH, conductivity, and dissolved oxygen were measured at three segments per stream using an Asko® multiparameter probe.

Current velocity and discharge were estimated using the approach of Craig (1987), with velocity being estimated by the formula: $V = \sqrt{2gD}$, where V = water velocity, g = gravity (9.8 m/s^2), and D = difference in the passage time at points D1 and D2 (D2-D1). Discharge was estimated by the formula: Discharge = stream width x stream depth x water velocity. These measurements were made at each sampling segment (five per stream) and averaged per stream.

Environmental integrity was evaluated for each stream using the Habitat Integrity Index (HII) of Nessimian et al. (2008). The HII varies from 0 (completely altered environments) to 1 (intact habitats) and has been widely used in ecological studies of aquatic insects in Brazil (Juen et al. 2014). All environmental variables are available in Supplementary 1.

4. *Spatial predictors*

Geographic coordinates of all streams were used to calculate spatial filters using a Euclidean distance matrix calculated with the "vegdist" function of the "vegan" package (Dray et al. 2016).

Spatial filters were calculated by Principal Coordinates of Neighbour Matrices (PCNM) with the minimum distance from the connectivity network being used as truncation distance (Dray et al. 2016). This technique makes it is possible to determine if there are spatial predictors structuring the distribution of communities. Other geographic and biotic processes (such as population growth, geographic dispersal, differential fertility or mortality, social organization, or competition dynamics) also can promote spatial autocorrelation and be captured by PCNM (Griffith and Peres-Neto 2006). Geographic coordinates were obtained using a Garmin Etrex handeld GPS. The PCNMs were calculated using the "pcnm" function of the "vegan" package (Oksanen et al. 2018). Subsequent selection of PCNMs was done using the "forward.sel" function of the "adhesive" package (Dray et al. 2016) in the program R.

5. *Data analysis*

Each stream was considered a sampling unit for data analysis, thus there was a total of 20 sampling units with 10 located in PNCM and 10 in PEM. The hypothesis that there would be no significant difference in genus richness between the two protected areas was tested by the Student's *t*-test using the "*t.test*" function of the basic "*stats*" package (R Core Team 2017). Richness of genera and abundance of individuals were the response variables and protected area was the categorical predictor with two levels, PNCM and PEM. Assumptions of normality and homogeneity of variance were tested, with the *t*-test for unequal variances being applied whenever these assumptions were not satisfied (Zar 2013).

Multivariate Permutational Analysis of Variance (PERMANOVA) was used to test the hypothesis that there was a significant difference in genus composition between the two protected areas (Anderson & Walsh 2013). This analysis was based on a matrix of genus composition and abundance data with Bray-Curtis distance as the response variable and protected area (PNCM or PEM) as the predictor variable. Significance was then determined by a Monte Carlo test with 9999 permutations (Anderson 2001; Anderson & Walsh 2013). A second PERMANOVA was run on the matrix of environmental variables using Euclidian distance as the response variable and protected area (PNCM or PEM) as the predictor variable, to determine whether significant differences existed between the protected areas in the set of environmental variables. The "bray" method was used to calculate Bray-Curtis distances in the "vegdist" function of the "vegan" package, while Euclidian distances were calculated using the "euclidean" method (Oksanen et al. 2018).

Two ordinations were constructed to graphically demonstrate differences in genera and environmental conditions between the two areas: Principal Coordinate Analysis (PCoA) using Bray Curtis distance for species composition with abundance data; and Principal Component Analysis (PCA) using Euclidean distance (Legendre & Legendre 1998) for standardized environmental variables

Given the requirement that there must be a minimum of 10 samples for each predictor used in models (Gotelli & Ellison 2004), and that the present study had 20 samples, variables needed to be selected (Forward Selection, Dray et al. 2016) to minimize residuals and produce more robust models.

Results

1. *Community structure*

A total of 1987 specimens were collected representing 46 genera and 15 families of the orders Ephemeroptera, Plecoptera and Trichoptera. The most common genera were *Anacroneuria*, *Smicridea*, *Leptonema*, and *Helicopsyche*, and the rarest were *Callibaetis*, *Macunahyphes*, *Fittkaulus*, *Homothraulus*, and *Massartella*, which were recorded only once each (Table 1).

2. *Description of environmental and spatial predictors*

None of the studied streams had an extremely low HII (<0.5), and so they all can be considered preserved or minimally altered (HI = 0.762 ± 0.084, mean ± standard deviation). The environmental variables differed significantly between the two study areas (Pseudo-F = 2.282, R2 = 0.112, p = 0.014). The first two axes of the PCA explained 58.4% of this environmental variation and partially separated the streams of PNCM and PEM (Fig 2). Forward selection did not select any good environmental predictors for community distribution. Six spatial filters were created to correct for spatial autocorrelation, with only PCNM 3 being selected as important for determining the spatial distribution of communities (PCNM 3: R^2= 0.092; F= 1.821; p= 0.021) (The graphical representation of the PCNM 3 in available in Supplementary 2).

3. *Differences in diversity patterns between protected areas*

Taxonomic richness of EPT genera did not differ significantly between study areas (t = -1.119, *p* = 0.2795), nor the abundance of individuals (t = 0.268, *p* = 0.791). A significant difference was found between the protected areas in the composition of EPT genera (Pseudo-F = 2.088, R^2 = 0.103, *p* = 0.015). The first two axes of the PCoA explained 50% of the variation in composition and partially separated streams of PNCM and PEM (Fig 3). Six genera were exclusive to PEM, nine genera were exclusive to PNCM and 31 occurred in both (Fig. 4).

Table 1. Genera of the orders Ephemeroptera, Plecoptera and Trichoptera collected in the streams of the Parque Nacional da Chapada das Mesas (PNCM) and Parque Estadual do Mirador (PEM).

Ordem	Família	Gênero	PNCM	PEM
Ephemeroptera	Baetidae	*Americabaetis* Kluge, 1992	12	0
		Callibaetis Eaton, 1881	1	0
		Criptonympha Lugo-Ortiz & McCafferty, 1998	1	1
		Waltzoyphius McCafferty & Lugo-Ortiz, 1995	3	1
		Zelusia Lugo-Ortiz & McCafferty, 1998	21	14
	Caenidae	*Caenis* Stephens, 1835	2	0
	Euthyplociidae	*Campylocia* Needham & Murphy, 1924	6	52
	Leptohyphidae	*Amanahyphes* Salles & Molineri, 2006	39	1
		Leptohyphes Eaton, 1882	1	2
		Macunahyphes Dias, Salles & Molineri, 2005	1	0
		Traverhyphes Molineri, 2001	1	3
		Tricorythodes Ulmer, 1920	12	2
	Leptophlebiidae	*Farrodes* Peters, 1971	55	32
		Fittkaulus Savage & Peters, 1978	1	0
		Hagenulopsis Ulmer, 1920	22	2
		Homothraulus Demoulim, 1955	0	1
		Hydrosmilodon Flowers & Dominguez, 1992	31	12
		Massartella Lestage, 1930	0	1
		Microphlebia Savage & Peters, 1983	0	2
		Miroculis Edmunds, 1963	19	43
		Paramaka Savage & Domínguez, 1992	10	3
		Simothraulopsis Demoulin, 1966	39	9
		Tikuna Savage, Flowers & Peters, 2005	3	0
		Ulmeritoides Traver, 1959	5	6
Plecoptera	Perlidae	*Anacroneuria* Klapálek, 1909	333	327
Trichoptera	Helicopsychidae	*Helicopsyche* Siebold, 1856	67	22
	Odontoceridae	*Marilia* Müller, 1880	7	8
	Hydropsychidae	*Leptonema* Guérin-Méneville, 1843	19	73
		Macronema Pictet, 1836	27	10
		Macrostemum Kolenati, 1859	10	21
		Smicridea McLachlan, 1871	96	150
		Synoestropsis Ulmer, 1905	0	8
	Hydroptilidae	*Flintiella* Agrisano,1995	0	8
		Hydroptila Dalman, 1819	3	0
		Metrichia Ross, 1938	2	3
		Neotrichia Morton, 1905	1	7
	Hidrobiosidae	*Atopsyche* Banks, 1905	2	0
	Glossosomatidae	*Mortoniella* Ulmer, 1906	0	10
	Leptoceridae	*Nectopsyche* Müller, 1879	5	16
		Oecetis McLachlan, 1877	17	13
		Triplectides Kolenati, 1859	10	8
		Gênero A Pes, 2005	6	39
	Philopotamidae	*Chimarra* Stephens, 1829	43	36
	Polycentropodidae	*Cernotina* Ross, 1938	14	63
		Cyrnellus Banks, 1913	6	18
		Polyplectropus Ulmer, 1905	7	0
TOTAL	**15**	**46**	**958**	**1029**

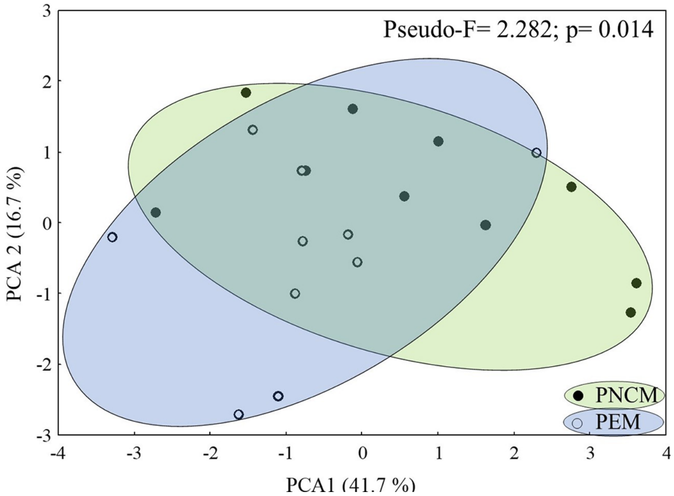

Figure 2. Ordination of the environmental variables in the streams of the Parque Nacional da Chapada das Mesas (PNCM) and Parque Estadual do Mirador (PEM).

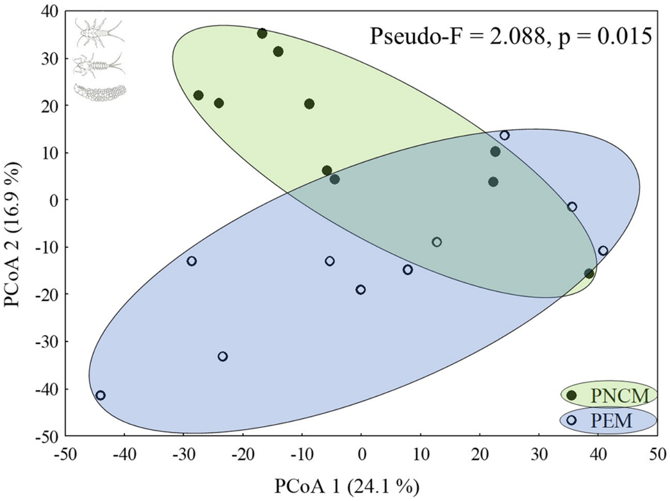

Figure 3. Ordination of the Ephemeroptera, Plecoptera and Trichoptera genera recorded in the streams of the Parque Nacional da Chapada das Mesas (PNCM) and Parque Estadual do Mirador (PEM).

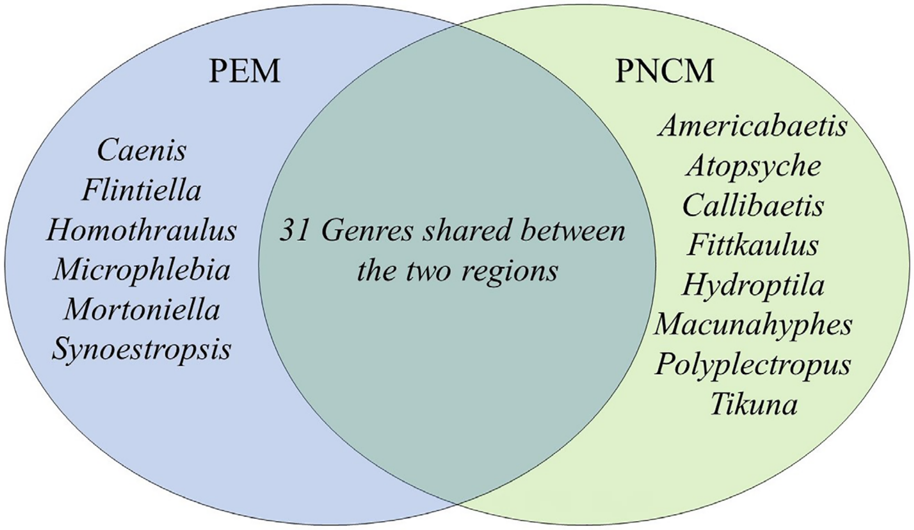

Figure 4. Diagram showing the genera collected exclusively in Parque Nacional da Chapada das Mesas (PNCM) and Parque Estadual do Mirador (PEM), and the genera shared by these two protected areas. PNCN is closest to the Amazon and PEM is in the most distant region.

Discussion

The hypothesis that the two protected areas would not differ in the richness of genera and abundance of individuals, but would differ in genus composition, was supported. Spatial distance and intrinsic characteristics, such as proximity to other biomes, appear to be the principal factors associated with the difference in taxonomic composition of the communities of the two areas. Parque Estadual do Mirador is further from Amazonia, whereas PNCM and is located more centrally within the Cerrado. This difference in the composition of communities of aquatic insects between the Cerrado-Amazonia transition and the central Cerrado is consistent with the findings of previous studies (Nogueira & Cabette 2011; Shimano et al. 2013; Juen et al. 2017).

Mass effect and neutral dynamics are the two ecological mechanisms most used to explain the spatial structuring of communities (Leibold et al. 2010). On a regional scale, such as the present study, Heino & Mykrä (2008) also found evidence of the importance of space in structuring aquatic insect communities, but in this case it was in a temperate region. Under a similar spatial configuration, Brasil et al. (2018) found the composition of adult odonate communities, a group of aquatic insects with greater dispersion potential than EPT, to be biogeographically congruent among different regions. Although integrity measured by the Habitat Integrity Index (Nessimian et al. 2008) has been the most important environmental predictor for EPT in Cerrado streams (Brasil et al. 2013; Brasil et al. 2014; Souza et al. 2011; Pereira et al. 2012; Nogueira et al. 2011), it varied little in the present study.

The association between EPT communities and environmental conditions has been well documented (Rosenberg & Resh 1993; Thorp et al. 2006; Wiens & Donoghue 2004). In the present study, none of the evaluated environmental variables were related to EPT communities. It seems likely that this situation reflects the fact that the studied stream environments were well-preserved or only lightly impacted (mean ± standard deviation HII = 0.76 ± 0.08). Water quality tends to be good and these environmental conditions vary little in preserved streams compared to anthropized streams (Martins et al., 2017). However, good water quality does reinforce the importance of the protected areas, given that previous studies have shown that deterioration of water quality in impacted areas adjacent to protected areas leads to changes in the communities found in anthropized streams (Faria et al. 2017; Montag et al. 2018). Decreased water quality due to anthropogenic impacts creates filters for the distribution of the different species (Martins et al. 2017), when there are anthropized.

The exclusive genera of PNCM and PEM may be a biogeographical signal. These results reinforce the importance of PNCM and PEM for aquatic biota conservation, especially the aquatic insects. The number of protected areas is currently declining worldwide (Ferreira 2014), and the Cerrado is the biome most impacted by agribusiness in Brazil (Lahsen 2016). Therefore, it is important that there are police and monitoring so that the "Parque Estadual do Mirador" and the "Parque Nacional da Chapada das Mesas" continue to play their role in conserving biodiversity in the future.

Supplementary material

The following online material is available for this article:

Table S1 - The complete presentation of environmental and spatial data for all streams in the two regions.

Figure S1 - Graphical representation of the spatial filter distribution by streams in the two regions.

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Conflicts of Interest

The authors have no conflict of interest.

Author Contributions:

Derise de Assunção Barbosa: Contribution to data collection; Contribution to manuscript preparation; Contribution to critical revision, adding intellectual content.

Leandro Schlemmer Brasil: Contribution to data analysis and interpretation; Contribution to manuscript preparation; Contribution to critical revision, adding intellectual content; Substantial contribution in the concept and design of the study.

Carlos Augusto Silva de Azevêdo: Contribution to manuscript preparation; Contribution to critical revision, adding intellectual content: substantial contribution in the concept and design of the study.

Lucas Ramos Costa Lima: Contribution to data collection; Contribution to data analysis and interpretation; contribution to critical revision, adding intellectual content; Substantial contribution in the concept and design of the study

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Non-marine invasive gastropods on Ilha Grande (Angra dos Reis, Rio de Janeiro, Brazil): distribution and implications for conservation

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Abstract: Invasive species are one of the main causes of biodiversity loss and the knowledge of their distribution is essential for conservation efforts. The present study inventoried the land and freshwater invasive molluscs of 25 villages of Ilha Grande (Rio de Janeiro, Brazil), an island with Atlantic Rainforest remnants. Three invasive species are found: *Melanoides tuberculata* (Müller, 1774), *Physa acuta* Draparnaud, 1805, both on two localities; and *Achatina fulica* Bowdich, 1822, on 16 localities. Most records were done on the island continental side, in anthropic areas, probably due to ease of access. One record was inside a conservation area, that calls concern about the spread of those species on the island and possible effects.

Keywords: *Achatina fulica, Melanoides tuberculata, Physa acuta, non-native species, Gastropoda.*

Moluscos continentais invasores na Ilha Grande (Angra dos Reis, Rio de Janeiro, Brasil): distribuição e implicação para a conservação

Resumo: As espécies invasoras são uma das maiores causas de perda de biodiversidade e o conhecimento acerca da sua distribuição é essencial para os esforços de conservação. O presente estudo inventariou os moluscos continentais invasores em 25 vilas da Ilha Grande (Rio de Janeiro, Brasil), uma ilha com remanescentes de Mata Atlântica. Três espécies invasoras foram encontradas: *Melanoides tuberculata* (Müller, 1774), *Physa acuta* Draparnaud, 1805, ambas em duas localidades; e *Achatina fulica* Bowdich, 1822, em 16 localidades. A maior parte dos registros foi no lado continental da ilha, provavelmente devido a facilidade de acesso. Um dos registros foi dentro de uma área de conservação, o que chama a atenção para a dispersão destas espécies na ilha, e seus possíveis efeitos.

Palavras-chave: *Achatina fulica, Melanoides tuberculata, Physa acuta, espécies não-nativas, Gastropoda.*

Introduction

The impact of non-native species on ecosystems is well discussed (e.g. Lockwood et al. 2005, Pimentel et al. 2005, Boltovskoy 2017, Cowie et al. 2017), especially on an insular system (Cowie 1998, Clavero & García-Berthou 2005, Pyšek et al. 2017). Some of these species, as *Achatina fulica* Bowdich, 1822, and *Melanoides tuberculata* (Müller, 1774), are dispersed worldwide, offering a combination of environmental, economic, and health concerns (Graeff-Teixeira 2007, Santos et al. 2012).

The African giant snail was introduced in Brazil around 1980 for commercial purposes and nowadays is widespread (Thiengo et al. 2007, Colley & Fischer 2009). This species is hermaphrodite, and a single specimen can initiate a new population, as autofecundation is possible (Thiengo et al. 2007, Fischer & Colley 2005). *Achatina fulica* can cause not only environmental problems, like the competition with native species, as *Megalobulimus* spp. (Gutiérrez-Gregoric et al. 2011), but also questions related to public health (Thiengo et al. 2007, Graeff-Teixeira 2007). *Achatina fulica* is the intermediate host for nematodes of medical and veterinary importance like *Angiostrongylus costaricensis* (Morera & Céspedes, 1971)*, Angiostrongylus cantonensis* (Chen, 1935), *Aelurostrongylus abstrusus* (Railliet, 1898), *Rhabditis* sp. and *Strongyluris* sp. (Vicente et al. 1993, Thiengo et al. 2008, 2010, Oliveira et al. 2010).

The Afro-Asiatic freshwater snail *M. tuberculata* was introduced in Brazil around 1967, in Santos, São Paulo state, probably by aquarium trade (Vaz et al. 1986). Nowadays it is widespread in Brazil (Fernandez et al. 2003, Santos et al. 2012, Miyahira et al. 2020). *Melanoides tuberculata* is parthenogenetic and resistant to desiccation (Facon et al. 2004; Weir & Salice 2012), two features that probably helped this species to disperse so widely. This species is the intermediate host of several trematodes, including *Centrocestus formosanus* (Nishigori, 1924) and *Philophthalmus gralli* Mathis & Leger, 1910 (Pinto & Melo 2011, Ximenes et al. 2017). There are no autochthonous human cases of these parasites described to Brazil, although it was already recorded in oriental immigrants, living in São Paulo city, infected with *Clonorchis sinensis* (Cobbold, 1875) (Leite et al. 1989).

Physa acuta is an invasive freshwater gastropod worldwide distributed (Paraense & Pointier 2003, Taylor 2003). The first record on Brazil was in 1966, under the name *Physa* (*Physella*) *cubensis* Pfeiffer, 1839 (Leme 1966), but nowadays it is widespread especially in southeastern Brazil (Santos et al. 2012) and some records in the states of Bahia and Goiás (Santos et al. 2016). This species is hermaphrodite and recognized for some environmental impacts (Taylor 2003, Zukowski & Walker 2009).

Ilha Grande is one of the largest insular remains of the Atlantic Rainforest in Brazil, and place of four conservation areas: Ilha Grande State Park, Praia do Sul Biological Reserve, Aventureiro Marine State Park, and Tamoios Ambiental Protection Area. The first two include almost all terrestrial surface of the island, except for most villages, that are included in Tamoios Ambiental Protection Area.

Those three invasive species were already recorded for Ilha Grande: *Achatina fulica* was first mentioned in 2002 (Santos et al. 2002), *M. tuberculata* in 2005 (Santos et al. 2007), and *P. acuta* in 2009 (Miyahira et al. 2010) to the main village of Ilha Grande, Vila do Abraão. However, the distribution of these species on the island is unknown. The main goal of this study was to present the distribution of these species on Ilha Grande, as also some observations on conservation issues, as the first step for future managing, conservation, and educational plans.

Material and Methods

Ilha Grande (23° 05', 23° 15' S and 44° 06', 44° 23' W), is an island located on the southern coast of the Rio de Janeiro state, southeast Brazil, far from 150 km from the city of Rio de Janeiro, in the municipality of Angra dos Reis. The island is covered with important remnants of Atlantic Forest especially on highlands (Callado et al. 2009), but near the coast, the habitats are modified by deforestation, non-native plants, domestic animals, and domestic sewage (Santos et al. 2007). The island can be divided into two sides, one facing the mainland (Continental or North side), with the most villages; and the other facing the open ocean (Oceanic or South side) with fewer villages and more difficult access by boats or trails.

Twenty-five localities were surveyed for invasive snails, were included the villages and nearby areas (anthropic and perianthropic). The localities were reached by trails or boats. Each locality was visited at least twice, and the search for the snails was done by three people at all kinds of suitable habitats. For *A. fulica* we did a visual inspection on walls, plants, under vegetation debris, and garbage for 30 minutes/person. *Melanoides tuberculata* and *P. acuta* were searched using a handled metallic scoop in freshwater streams for 30 minutes/person. At each village were recorded the "presence" or "absence" regarding the occurrence of the target invasive species: *A. fulica, M. tuberculata*, and *P. acuta*. All collecting stations were georeferenced using a Garmin Etrex. Samples of live molluscs were placed in plastic bags or pots and took back to the laboratory for biological observations, taxonomic identification, and further parasitological studies.

Voucher specimens are housed at the Malacological Collection of the Universidade do Estado do Rio de Janeiro (UERJ).

Results

Sixteen localities of Ilha Grande present at least one invasive mollusc species: *M. tuberculata*, *P. acuta,* or *A. fulica* (Figure 1 and Table 1). In most localities only *A. fulica* was found; in Praia Vermelha, two species were recorded (*M. tuberculata* and *P. acuta*), and on Vila do Abraão, all the three species occurs. There are only two villages with invasive species on the oceanic side (Provetá and Vila Dois Rios), and 14 on the continental side (Figure 1 and Table 1). All the records of freshwater invasive molluscs were on the continental side, on only two villages (Vila do Abraão and Praia Vermelha). *Achatina fulica* has distributed along 16 (64%) villages: 14 on the continental side and two on the oceanic side. *Achatina fulica* was found colonizing not only the urban environments but also the surroundings of the villages in areas of secondary Atlantic Forest. The two freshwater invasive gastropods were found only at impacted water bodies, *M. tuberculata* in sandy bottoms, and *P. acuta* attached to aquatic vegetation. The two species also colonize debris and/or man-made habitats.

Most of the records were done on the borders of Ilha Grande State Park (Fig. 1), except for the occurrence of *A. fulica* in Vila Dois Rios that is inside the park. On Praia do Sul Biological Reserve no invasive molluscs were found.

Discussion

Achatina fulica was widespread on villages of Ilha Grande (Figure 1 and Table 1), this is related to some features of this species like

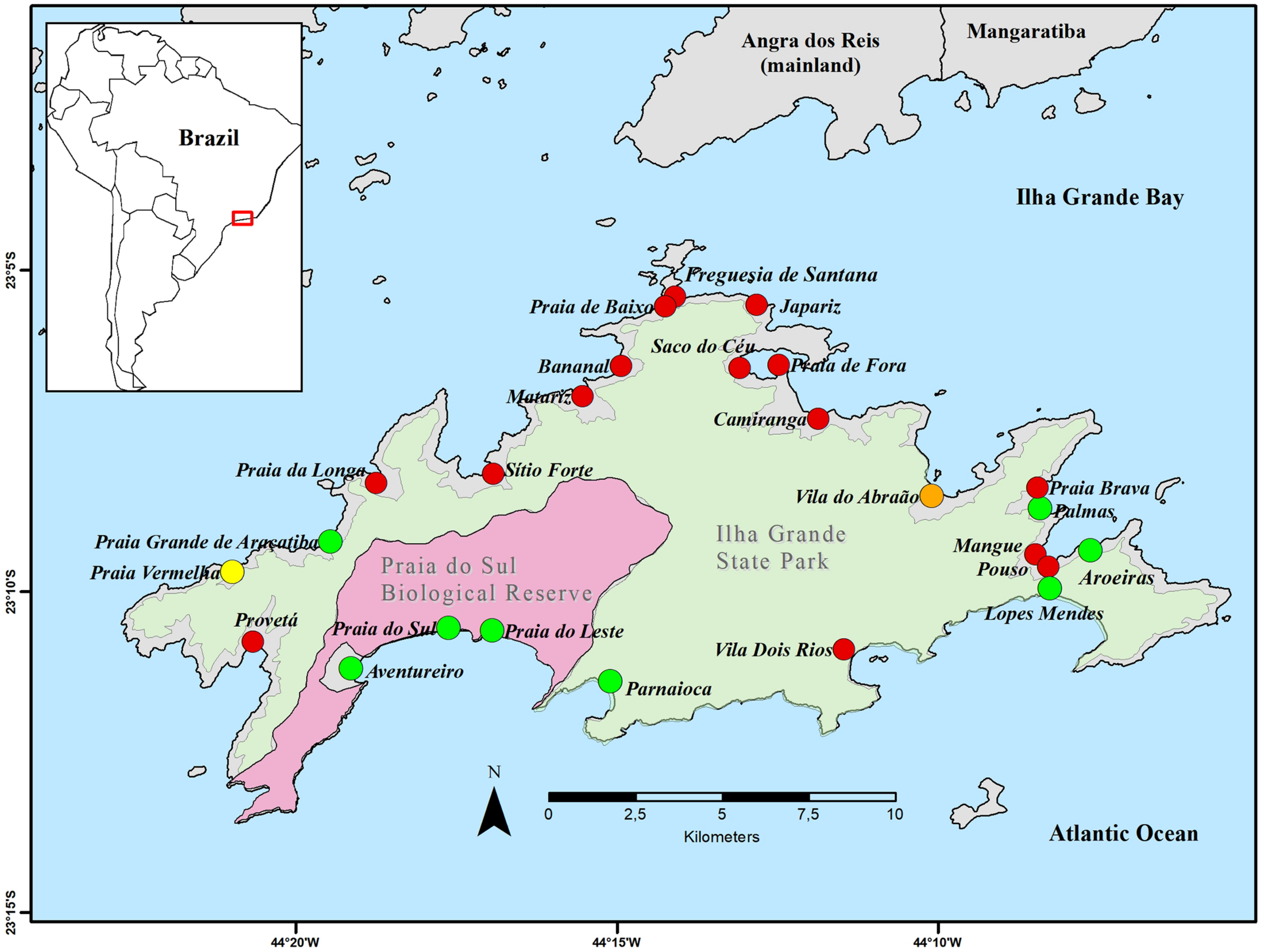

Figure 1. Distribution of *Achatina fulica*, *Melanoides tuberculata*, *and Physa acuta* in several villages of Ilha Grande. Red dots – *Achatina fulica*; Yellow dots – *Melanoides tuberculata* and *Physa acuta*; Orange dots – *A. fulica, M. tuberculata*, and *P. acuta*; and Green dots – Invasive molluscs not found.

hermaphroditism, a high number of egg-laying per year (3 to 4), a high number of eggs per laying episode (200 to 400), and high adaptability to environmental conditions (Kosloski et al. 2002, Fischer & Colley 2004). This species was probably dispersed on Ilha Grande attached to plants and other goods. The distribution of *A. fulica* on Ilha Grande seems to be restricted to anthropic and nearby areas of secondary forest. The species was so adapted to the anthropic environment of Ilha Grande that were found at gardens, walls, and streets. Fischer et al. (2008) stated that these urban areas offer resting sites and shelters against predation. Other authors already observed these environmental preferences of *A. fulica* (Colley & Fischer 2009, Goldyn et al. 2017). The urban population can expand to nearby preserved forests (Raut & Baker 2002, Fischer et al. 2006, Faraco 2011). Ilha Grande has a rich and diverse fauna of land snails (Haas 1953, Santos & Monteiro 2001, Santos et al. 2010, Nunes & Santos 2012, Cuezzo et al. 2018), but few occur in anthropic habitat, as preferred by *A. fulica*. However, the expansion of this species to preserved areas can increase the negative impacts of giant African land snail on the island; although some studies do not show so drastic outputs (Miranda et al. 2015, Miranda & Pecora 2017, O'Loughlin & Green 2017).

Melanoides tuberculata and *P. acuta* were restricted to two localities of Ilha Grande (Figure 1 and Table 1); the first record of *M. tuberculata* was in 2005 in Vila do Abraão (Santos et al. 2007) and *P. acuta* was in 2009 (Miyahira 2010) in the same locality. The population of *M. tuberculata* at Vila do Abraão was initially found in low numbers (Santos et al. 2007), therefore the population increased, also affecting negatively the native fauna (Braga et al. 2014). A subsequent record of *M. tuberculata* was done at Praia Vermelha (Gonçalves et al. 2012) in low numbers, probably indicating a recent introduction event, but the population still present on the following collecting events, probably indicating that population was established. This last locality is far from Vila do Abraão, therefore it is probable that this distribution extension was human-mediated (e.g. discharge of aquarium water with aquatic plants and snails). The aquatic species need more factors to be transported from one place to another, resulting in a slower dispersion, and narrow distributions on Ilha Grande. Another factor that possibly restricts the distribution of aquatic invaders is the morphology of the streams of Ilha Grande, usually small and running across small stretch before reaches the sea. Thus, these are streams with a low amount of nutrients and conductivity, preventing some newly introduced species to

Table 1. The surveyed localities of Ilha Grande (Angra dos Reis, Rio de Janeiro, Brazil) and the date of first sighting of the three invasive species. N.F.= Not Found.

	Localities	GPS	Achatina fulica	Melanoides tuberculata	Physa acuta
Continental/North side	Vila do Abraão	23°08’49.5”S 44°10’13.4”W	Jan-2002	Aug-2005	Feb-2009
	Camiranga	23°07’17.81”S 44°11’56.39”W	Aug-2008	N.F.	N.F.
	Praia de Fora	23°06’48.48”S 44°12’19.14”W	Aug-2008	N.F.	N.F.
	Saco do Céu	23°06’37.87”S 44°13’00.39”W	Aug-2008	N.F.	N.F.
	Japariz	23°05’34.36”S 44°12’48.79”W	Aug-2008	N.F.	N.F.
	Freguesia de Santana	23°05’22.34”S 44°14’06.85”W	Sep-2007	N.F.	N.F.
	Praia de Baixo	23°05’30.0”S 44°14’13.7”W	Aug-2008	N.F.	N.F.
	Bananal	23°06’20.37”S 44°14’51.77”W	2004	N.F.	N.F.
	Matariz	23°06’58.96”S 44°15’31.35”W	2004	N.F.	N.F.
	Sítio Forte	23°07’31.68”S 44°16’25.85”W	Sep-2006	N.F.	N.F.
	Praia da Longa	23°08’17.13”S 44°18’39.16”W	Aug-2008	N.F.	N.F.
	Praia Grande de Araçatiba	23°09’11.95”S 44°19’20.67”W	N.F.	N.F.	N.F.
	Praia Vermelha	23°09’41.08”S 44°20’57.62” W	N.F.	Jun-2009	Jun-2009
	Mangue	23°09’35.36”S 44°08’14.12”W	Aug-2008	N.F	N.F.
	Aroeiras	23°09’21.49”S 44°07’38.39”W	N.F.	N.F.	N.F.
	Pouso	23°09’30.19”S 44°08’25.68”W	Aug-2008	N.F.	N.F.
	Palmas	23°08’46.21”S 44°08’21.82”W	N.F.	N.F.	N.F.
	Praia Brava	23°08’22.12”S 44°08’26.41”W	Aug-2008	N.F.	N.F.
Oceanic/South side	Praia do Leste	23°10’35.5”S 44°17’05.7”W	N.F	N.F.	N.F.
	Praia do Sul	23°10’36.7”S 44°18’03.1”W	N.F	N.F.	N.F.
	Provetá	23°10’48.29”S 44°20’34.34”W	Mar-2006	N.F.	N.F.
	Aventureiro	23°11’10.43”S 44°19’06.96”W	N.F.	N.F.	N.F.
	Parnaioca	23°11’28.73”S 44°15’02.32”W	N.F.	N.F.	N.F.
	Vila Dois Rios	23º11’01.8”S 44° 11’ 63.5”W	Sep-2006	N.F.	N.F.
	Lopes Mendes	23°10’21.32”S 44°07’29.61”W	N.F.	N.F.	N.F.

establish, and the dispersion of the exiting species to other places. The shell morphology of *M. tuberculata* is also different in both populations (Gonçalves et al. 2012). *Physa acuta* occurs at the same stream of *M. tuberculata* in Vila do Abraão and Praia Vermelha. This pulmonate snail was the last invader to be found on the island, and the impacts of this species over native fauna are scarce on the literature but cannot be neglected (Zukowski and Walker 2009). Gonçalves et al. (2014) reported *Omalonyx matheroni* (Potiez & Michaud, 1835) in Villa do Abraão, but the introduction was not successful after periodic site visits (personal communication).

Most records of invasive molluscs in Ilha Grande were done on the North (or continental) side, facing the mainland. The two sides of the island present different environmental conditions (Nunes & Santos 2012) but it is probably that observed distribution is not related to those environmental factors. On the North side, there is a large number of villages, and an easier movement of goods and people, thus increasing the likelihood of an introduction. Vila do Abraão, the village with a greater flow of people on the island, was the unique place where the three invasive species were found together. The two villages on the oceanic side that presented invasive species were Provetá and Vila Dois Rios. Provetá has the second-largest resident population of the island, and Vila Dois Rios is connected by road to Vila do Abraão, one of the few roads on the island. Thus, the uncontrolled movement of people and goods seems to be important to understand and mitigate the spread of invasive molluscs on the island. This phenomenon was probably related to the explosive increase of touristic activities after the closing of Ilha Grande's Prison in 1994 (Prado 2003, Cadei et al. 2009), although a previously detailed inventory of non-marine molluscs is not available. Haas (1953) pointed out some species of land and freshwater molluscs of Ilha Grande, and at that time, no non-native species were reported.

In addition to the tourism, the villages have experienced population growth and urban expansion, this can easily be noted at Vila do Abraão, Provetá, Bananal, Matariz, and Longa. In these villages there were always new buildings that demand a continuous trade of goods with the mainland, increasing the risk of unwanted introduction of species. Moreover, Ilha Grande doesn't have any kind of sanitary barrier; villagers and tourists can bring almost all kinds of animals and plants to the island. This lack of inspection can be a highway to the introduction of non-native species. The eight villages that do not present invasive species on Ilha Grande, also do not present a great touristic flow, except for Palmas.

Most records of invasive molluscs on Ilha Grande were out of restrictive conservation areas, except for *A. fulica* inside the Ilha Grande State Park.

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This record inside the park and those on the borders of conservation areas causes concern and calls attention to the need for inspection on the goods transported to the island. Those three invaders can expand their distribution on the island if the proper conditions were offered.

Another important consequence of the dispersion of invasive species is the introduction and dispersal of parasites (Font 2003). Oliveira & Santos (2019) recorded cysts of the nematode *Strongyluris* Müller, 1894 in the pallial system of *A. fulica* collected in two populations from Ilha Grande: Vila do Abraão and Vila Dois Rios. This parasite has as final host lizards or rarely anurans (Vicente et al. 1993, Bursey et al. 2003), although *A. fulica* can also harbor other helminths that have the man as the final common host (Thiengo et al. 2007). Some habitat of this species, like coprophagy, can make easy the establishment of new cycles (Mead 1961, Fischer 2009, Goldyn et al. 2017). Ximenes et al. (2017) reported the occurrence of *Centrocestus formosanus* (Nishigori, 1924) in *M. tuberculata* in the population from Vila do Abraão, a helminth that has birds as the final hosts.

The occurrence of *A. fulica*, *M. tuberculata,* and *P. acuta* represent a risk to conservation of native species and public health, an effort to control those species must be done together with environmental education, public health, and environmental authorities to prevent and mitigate the spread of these invasive species on Ilha Grande.

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Author Contributions

Jaqueline L. de Oliveira: Substantial contribution in the concept and design of the study; Contribution to data collection; Contribution to data analysis and interpretation; Contribution to manuscript preparation; Contribution to critical revision, adding intelectual content.

Igor C. Miyahira: Substantial contribution in the concept and design of the study; Contribution to data collection; Contribution to data analysis and interpretation; Contribution to manuscript preparation; Contribution to critical revision, adding intelectual content.

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Sonia B. dos Santos: Substantial contribution in the concept and design of the study; Contribution to data collection; Contribution to manuscript preparation; Contribution to critical revision, adding intelectual content.

Conflicts of interest

The authors declare that they have no conflict of interest related to the publication of this manuscript.

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Article

Diet and histological features of digestive tube from four discarded fish species by trawl bycatch in Northeastern Brazil

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BOMFIM, A.C., FARIAS, D.S.D., MOURÃO-JUNIOR, H.B., MORAIS, I.C.C., ROSSI, S., GAVILAN, S.A., SILVA, F.J.L. **Diet and histological features of digestive tube from four discarded fish species by trawl bycatch in Northeastern Brazil**. Biota Neotropica 20(3): e20200951. https://doi.org/10.1590/1676-0611-BN-2020-0951

Abstract: Fish feeding habit associated to the morphology of their digestive tube can provide useful biological and ecological information. Between January and December 2012, we examined 16 fish species discarded by trawl in the Potiguar basin, northeastern Brazil, and classified them into four groups according to Alimentary Index – IAi. Our aims were to: (1) provide information on diet composition of one species represented by each group (*Opisthonema oglinum, Conodon nobilis, Larimus breviceps* and *Sphoeroides testudineus*), (2) examine the morphology of their digestive tube, and (3) associate ingested food items with the morphological features. Stomach contents were collected and classified according to IAi: main, accessory and secondary categories; fragments of esophagus, stomach, and small intestine were examined using paraffin section technique. Biometric data (total length-TL, standard length-SL and total weight-TW), stomach weight-SW, and gonadal weight-GW were recorded and applied in two indices: (1) the stomach Repletion Index - SRI (SRI = SW/TW × 100) and (2) the gonadosomatic index – GSI (GSI = GW/TW × 100) for the analyses of feeding and reproductive activities, respectively. The main food categories were (1) Teleostei for C. *nobilis*, (2) Crustacea for *L. breviceps* and *O. oglinum*, and (3) Mollusca for S. testudineus. The feeding activity was higher during the dry season, while the GSI was higher in the rainy season. The morphological analysis revealed a regular structure of the tissues analyzed. We highlight the presence of tubular gastric glands and strong muscular walls in the stomach (*O. oglinum*; *L. breviceps*); well-developed pyloric caecum in the intestine (*O. oglinum*; *C. nobilis*; *L. breviceps*); and presence of pseudo-stomach (*S. testudineus*). Our study confirms that the digestive tube of the examined species has morphological adequacy for carnivorous feeding habit, providing useful information on tissue structures associated to diet composition.

Keywords: *feeding ecology; fish; histology; feeding activity.*

Dieta e características histológicas do tubo digestivo de quatro espécies de peixes descartadas pela pesca de arrasto no nordeste do Brasil

Resumo: O hábito alimentar de peixes associado à morfologia do tubo digestivo pode fornecer informações biológicas e ecológicas úteis. Entre janeiro e dezembro de 2012, examinamos 16 espécies de peixes descartadas por pesca de arrasto na Bacia Potiguar, nordeste do Brasil, e as classificamos em quatro grupos, de acordo com o Índice Alimentar-IAi. Nossos objetivos foram: (1) fornecer informações sobre a composição da dieta de uma espécie representante de cada grupo (*Opisthonema oglinum, Conodon nobilis, Larimus breviceps e Sphoeroides testudineus*), (2) examinar a morfologia de seu tubo digestivo e (3) associar os itens alimentares ingeridos com as características morfológicas descritas. O conteúdo estomacal foi coletado e classificado de acordo com o IAi nas categorias: principal, acessória e secundária; fragmentos de esôfago, estômago e intestino delgado foram examinados pela técnica de secção em parafina.

Os dados biométricos (comprimento total-CT, comprimento padrão-CP e peso total-PT), peso do estômago-PE e peso gonadal-PG foram registrados e aplicados em dois índices: (1) Índice de Repleção Estomacal - IR (IR = PE / PT × 100) e (2) Índice Gonadossomático - IGS (IGS = PG / PT × 100) para análises de atividade alimentar e reprodutiva, respectivamente. As principais categorias alimentares foram: (1) Teleostei para *C. nobilis*, (2) Crustacea para *L. breviceps* e *O. oglinum*, e (3) Mollusca para *S. testudineus*. Maior atividade alimentar ocorreu durante a estação seca, e o IGS foi maior na estação chuvosa. O exame morfológico revelou estrutura regular dos tecidos analisados, destacando-se a presença de glândulas gástricas tubulares e camadas musculares espessas no estômago (*O. oglinum*; *L. breviceps*); cecos pilóricos bem desenvolvidos no intestino (*O. oglinum*; *C. nobilis*; *L. breviceps*); e presença de pseudoestômago (*S. testudineus*). Nosso estudo confirma que o tubo digestivo das espécies examinadas possui adequação morfológica ao hábito alimentar carnívoro e fornece informações úteis sobre as estruturas dos tecidos associados à composição da dieta.

Palavras-chave: *ecologia alimentar; peixe; histologia; atividade alimentar.*

Introduction

The coast in Rio Grande do Norte State, Brazil, stretches over approximately 400 km, and is characterized by significant fishing activity with 76.5% represented by artisanal fisheries (Ibama 2010). Thus, the discard of trawl fishing is concerning and relevant for marine conservation (Helfman et al. 2009). Fish bycatch is often higher than capture of target species, especially in multi-target small-scale fisheries operating in the northwestern coast of Rio Grande do Norte (Silva-Júnior et al. 2013). In this region, trawl fisheries conducted by small boats aim to capture shrimp; however, many fish are accidentally captured (Bomfim et al. 2019).

Over the last decades, concerns about bycatch of charismatic species such as dolphins and marine turtles have increased and quickly expanded to other species (e.g. juvenile fish discarded by bottom trawls) (Kennelly & Broadhurst 2002, Burgess et al. 2018). Bycatch of juvenile animals influences their conservation and affect density and distribution of predators, resulting in a limited abundance and distribution (Block et al. 2011, Stewart & Jones 2001).

Fishes feed on various resources at different water depths and studies on digestive tube descriptions provide useful biological and ecological information on environmental factors and food availability (Canan et al. 2012). Aspects of the digestive tube morphology have been described in several fish species, increasing the interest on their morphological variation, diet, and behavior (as a result from trophic levels in the food web and the high ichthyofaunal diversity) (Vieira-Lopes et al. 2013). However, few studies have investigated anatomical and histological aspects of neotropical ichthyofaunal digestive tube (Canan et al. 2012). The analyses of feeding habits and morphology features provide knowledge on food dynamics (e.g. intra- and inter-specific interactions and community energy flux) and habitat use (Hajisamaea et al. 2003; Cachera et al. 2017).

Most tropical fishes have an intense trophic plasticity and varied diet, which may be related to spatial, seasonal, ontogenetic, and behavioral factors, providing more advantages to generalist than to specialist species (Lowe-McConnell 1999, Abelha et al. 2001). We examined and classified 16 fish species into four groups, according to Alimentary Index (IAi), discarded by trawls in the study site. Our study aimed to (1) provide information on diet composition of one species represented by each group (*Opisthonema oglinum*, *Conodon nobilis*, *Larimus breviceps* and *Sphoeroides testudineus*), (2) examine the morphology of their digestive tube, and (3) associate ingested food items to morphological features.

Materials and Methods

1. *Study site*

This study was carried out in northeastern Brazil, between the municipalities of Caiçara do Norte (5 ° 4’1 .15 “S, 36 ° 4’36 .41” W), Rio Grande do Norte State (RN), and Icapui (4°38’48.28”S and 37°32’52.08” W), Ceará State (CE), a region known as the Potiguar Basin, which stretches over approximately 300 km (Figure 1). The study site is formed by crystalline basement rocks (Soares et al. 2003), with different geomorphological and environmental characteristics along the extension monitored. In Icapuí, tourism, artisanal fishing, and saliniculture are the main economic activities, while in Caiçara do Norte, artisanal or professional fishing (depending on the beach), wind energy, and gas/petroleum exploration are the local economic activities. The climate in the region is semi-arid with varied humidity, low rainfall, and two well-defined seasons: dry (between August and December), with strong winds, and rainy (from January to July) (Jimenez et al. 1999, Testa & Bosence 1999).

Since 2010, the Projeto Cetáceos da Costa Branca - Universidade do Estado do Rio Grande do Norte (PCCB-UERN) has conducted the Beach Monitoring Program in the Potiguar Basin (Programa de Monitoramento de Praias da Bacia Potiguar – PMP-BP). The PMP-BP is part of an environmental constraint compliance enforced by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis - IBAMA (*Brazilian Institute of the Environment and Renewable Natural Resources*) for oil exploration by PETROBRAS (Petróleo Brasileiro S.A.; agreement number 2500.005657510.2).

2. *Sampling*

Between January and December 2012, samples were collected monthly during daily monitoring conducted by a trained field team using a four-wheeled (i.e. quad) motorcycle and a portable GPS, and prior approaches with fishermen at the time of drag. Trawl nets varied from 6 to 40 m long X 3 m wide, and were usually made of mono or braided nylon (also named “trammel”). Fishermen often use this type of trawl net to capture shrimp during the day, at an average depth of 1.5 m; however, other animals are also caught by the net.

The individuals collected were stored in isothermal bags and transported to the support base, where they were photographed, fixed in 10% formalin, and preserved in 70% alcohol for further identification by experts and according to previous studies (Menezes & Figueiredo

2000, Szpilman 2000, Júnior et al. 2010). We evaluated total length-TL, standard length-SL, total weight-TW, stomach weight-SW, and gonadal weight-GW.

3. Diet analyses

The stomach contents were preserved in 70% alcohol and the analyses combined three methods: (1) Points method: visual estimate of volume percentage of each food item. Different classes were given a number of points and the sums of points for each food item were reduced to percentages to show the percentage of diet composition (Swynnerton & Worthington 1940). (2) Frequency of occurrence (F%): number of stomachs containing one or more individuals of each food category was expressed as a percentage of all stomachs containing food (Dineen 1951, Dunn 1954, Kennedy & Fitzmaurice 1972). (3) Volumetric method by direct estimation (V%): the measurement of each food item or group of items sorted from the stomach contents was performed in a graduated measuring device, according to Wolfert & Miller (1978), considering the volume of each food item and expressed as percentage of all food item (Kawakami & Vazzoler 1980). Microscopic items were analyzed through slides prepared with the homogenized food contents.

Alimentary Index (Índice Alimentar - IAi) (Kawakami & Vazzoler 1980) was applied for each food item: $IAi = Fi \times Vi / \sum n(Fi \times Vi)$; whereas F = Frequency of occurrence (%) of each item, and V = Volume (%) of each item, and i= 1,2,… n= determined food item. In order to analyze the diet composition considering the IAi, food items found were classified into categories according to Rosecchi & Nouaze (1987): $IAi < 0.25$ (accessory category), $0.25 \leq IAi < 0.5$ (secondary category), and $IAi \geq 0.5$ (main category).

4. Feeding and reproductive activities

Feeding and reproductive activities were analyzed considering the rainy and dry seasons. Climatic, geomorphologic, phytogeographic, and tectonic differences can influence wind direction and ocean circulation pattern (Vital 2006), changing food availability and fish reproductive behavior. The data on precipitation for the study period was obtained from Proclima (Real-Time Climate Monitoring Program for the Northeast) (http://www6.cptec.inpe.br/proclima/ accessed in January 31st 2013).

The feeding activity was calculated using the Stomach Repletion Index (SRI): $SRI = SW/TW \times 100$ that provides information on the influence of spatial-temporal variation in the feeding activity (Stricta-Pereira et al. 2010).

The gonadosomatic index (GSI) was used to determine the reproductive activity: $GSI = GW/TW \times 100$, which is an indicator of gonadal functional status (Maddock & Burton 1999, Wootton et al. 1978).

5. Tissue processing

Samples of the digestive tube were obtained during postmortem examination, fixed in 10% formalin, dehydrated, diaphanized, and embedded in paraffin. Serial 5 µm sections were prepared and stained with hematoxylin and eosin (HE).

6. Statistical analyses

The Cluster analysis, considering Euclidean distance as a measure, was performed to evaluate similarity among fish species according to types of food items found in their stomach contents. The Wards method was applied to organize the variables into four groups. The dendrogram generated from similarity of ingested food items was used to analyze the relation of morphohistologic features of the digestive tube with food habit for one species from each group.

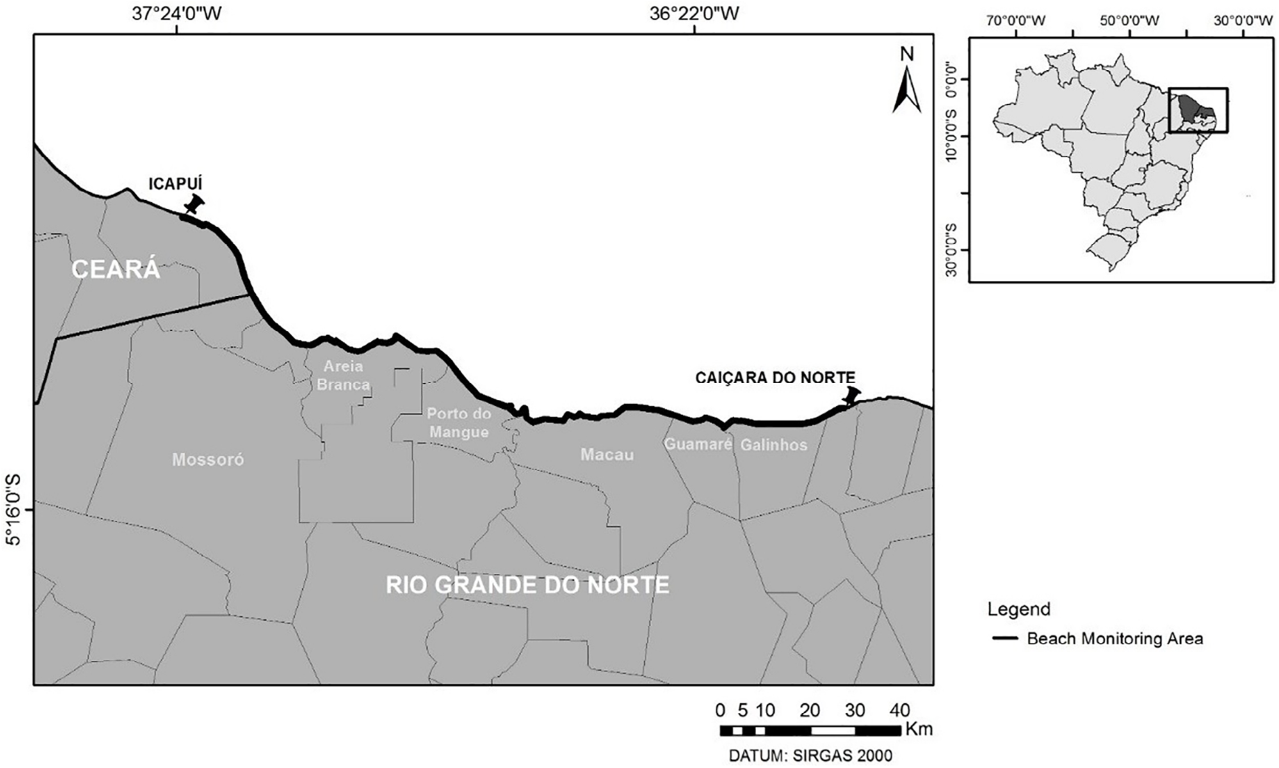

Figure 1. Geographic distribution of the study site, Brazilian northeastern coast. Source: Projeto Cetáceos da Costa Branca - Universidade do Estado do Rio Grande do Norte (PCCB-UERN).

Results

We examined and classified 1,232 individuals into five orders, 11 families, and 16 species. *Pomadasys corvinaeformis* (n = 423; 34.33%; Roughneck grunt; Perciformes: Pomadasyidae), *Opisthonema oglinum* (n = 136; 11.04%; Atlantic thread herring; Clupeiformes: Clupeidae) and *Larimus breviceps* (n = 129; 10.47%; Shorthead drum; Perciformes: Sciaenidae) represented more than 50% of individuals captured, followed by *Menticirrhus littoralis* (n = 98; 7.95%; Gulf kingcroacker; Perciformes: Sciaenidae), *Conodon nobilis* (n = 93; 7.55%; Barred grunt; Perciformes: Haemulidae), *Pelona harroweri* (n = 70; 5.68%; American coastal pellona; Clupeiformes: Pristigasteridae), *Lycengraulis grossidens* (n = 52; 4.22%; Atlantic sabretooth anchovy; Clupeiformes: Engraulidae), *Selene brownii* (n = 51; 4.14%; Caribbean moonfish; Perciformes: Carangidae), *Aspistor luniscutis* (n = 50; 4.06%; Sea catfish; Siluriformes: Ariidae), *Bagre marinus* (n = 40; 3.25%; Gafftopsail sea catfish; Siluriformes: Ariidae), *Sphoeroides testudineus* (n = 34; 2.76%; Checkered puffer; Tetraodontiformes: Tetraodontidae), *Polydactylus virginicus* (n = 28; 2.27%; Barbu; Perciformes: Polynemidae), *Bagre bagre* (n = 11; 0.89%; Coco sea catfish; Siluriformes: Ariidae), *Gymnura micrura* (n = 8; 0.65%; Smooth butterfly ray; Myliobatiformes: Gymnuridae), *Lagocephalus laevigatus* (n = 5; 0.41%; Smooth puffer; Tetraodontiformes: Tetraodontidae), and *Dasyatis guttata* (n = 4; 0.32%; Longnose stingray: Myliobatiformes: Dasyatidae).

1. *Diet composition*

We analyzed the diet of 16 species discarded by trawl, which were classified into four groups, according to IAi: (I) Group represented by species with 'Crustacea' as the main food category, 'Sediment' as secondary or accessory category: *L. grossidens* and *O. oglinum*; (II) Group represented by species with 'Teleostei' as the main food category: *C. nobilis*, *B. bagre*, *B. marinus* and *G. micrura*; (III) Group represented by species with 'Crustacea' as the main food category, 'Teleostei' as secondary or accessory category: *P. virginicus*, *M. littoralis*, *S. brownii*, *D. guttata*, *L. breviceps*, *P. harroweri*, *A. luniscutis* and *P. corvinaeformis*; and (IV) Group represented by species with 'Mollusca' as the main food category and 'Echinodermata' as accessory category: *L. laevigatus* and *S. testudineus* (Figure 2).

Stomach contents from 222 individuals of four species representing each group were fully analyzed: group I – *O. oglinum* (n = 85); group II – *C. nobilis* (n = 51); group III – *L. breviceps* (n = 67), and group IV - *S. testudineus* (n = 19). In addition, we analyzed stomach contents of *M. littoralis* (n = 33) and *P. corvinaeformis* (n = 52), species most discarded by trawl, along with *C. nobilis* and *L. breviceps*.

Food items were found and classified into 15 categories: (1) Nematoda, (2) Cnidaria, (3) Mollusca, (4) Polychaeta, (5) Crustacea, (6) Insecta, (7) Echinodermata, and (8) Teleostei (Animal categories); (9) Algae; (10) Vegetal matters and (11) Animal matters (semi digested material); (12) Sediment; (13) Rocks; (14) Plastic matters and (15) Non-identified items (due to advanced digestion stage of food items).

O. oglinum had the higher number of food items (n = 27). Gastrophoda (Mollusca category) represented the higher frequency of occurrence (40%), followed by Dendrobranchiata (Crustacea category) (25.882353%), sediment (20%) and Cirripedia (Crustacea category) (20%). According to the IAi, Crustacea represented the main food category (0.509717). Sediment was classified as secondary category (0.252846), and Mollusca had the highest value of all accessory categories (0.201009) (Table 1).

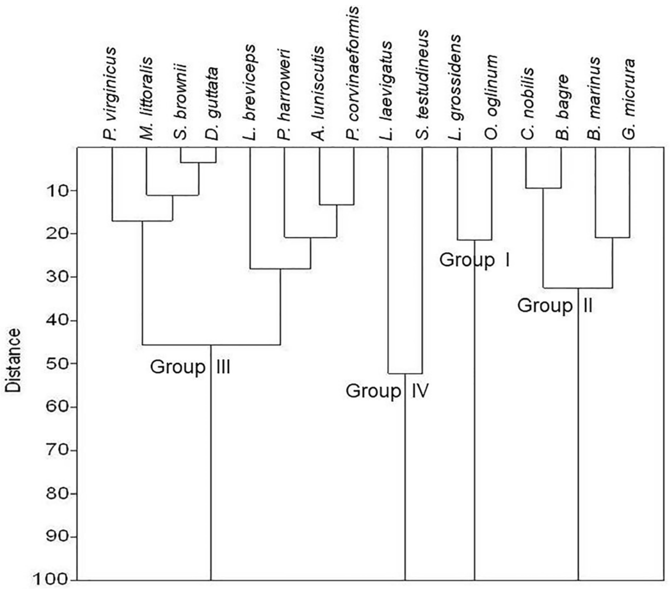

Figure 2. Dendogram of 16 species discarded by trawl in northeastern Brazil between January and December 2012, based on similarity of ingested food items.

C. nobilis: whole fish (Teleostei category) represented the highest frequency of occurrence (76.470548%), followed by Dendrobranchiata (19.607843%). Fourteen food items were described, and the Teleostei represented the main food category (IAi= 0.877130). Crustacea, Animal matters, Vegetal matters, and Mollusca were considered accessory categories, and Crustacea presented the highest value (0.111814) (Table 1).

M. littoralis: Dendrobranchiata was the item with the highest frequency of occurrence (57.575758%), followed by Animal matters (30.303030%). Twelve food items were described, and according to the IAi, Crustacea category (0.698015) mainly composed the diet. Although Animal matters, Polychaeta, Mollusca, Teleostei, and Cnidaria were all considered accessory categories, Animal matters represented the highest index (0.146784), followed by Polychaeta (0.118038) (Table 1).

L. breviceps had 15 food items described, with Dendrobranchiata representing the highest frequency of occurrence (77.611940%), followed by fry (28.358209%), and rest of Teleostei (23.880597%) items. This species presented high diversity of food items and Crustacea was the main category (IAi= 0.836622). Teleostei (0.154072), Mollusca (0.008634), and Sediment (0.000692) were accessory categories, and Teleostei had the highest index (Table 1).

P. corvinaeformis: the highest frequency of occurrence was Dendrobranchiata (36.538462%), followed by rest of Teleostei (32.692305%). Fourteen food items were described, and Crustacea represented the main food category (IAi= 0.512109); Teleostei and Mollusca had the highest values of IAi (0.171408 and 0.153332, respectively) compared to other accessory categories (Animal matters, Sediment, Polychaeta, Insecta, Echinodermata and Algae) (Table 1).

S. testudineus showed the least diversity of food items (n = 8). Bivalvia represented the highest frequency of occurrence (100%), followed by Clypeasteroida (Echinodermata category) (50%). Regarding the IAi, Mollusca was the main food category (0.779386). Echinodermata, Sediment, Teleostei, Non-identified items, Vegetal matters, and Crustacea were accessory categories (Table 1).

Table 1. Alimentary Index (IAi) of food items found in the stomach contents of six fish species discarded by trawl in northeastern Brazil from January to December 2012.

Food Items classified into fifteen categories	***Opisthonema oglinum*** **(n = 85)** **IAi**	***Conodon nobilis*** **(n = 51)** **IAi**	***Menticirrhus littoralis*** **(n = 33)** **IAi**	***Larimus breviceps*** **(n = 67)** **IAi**	***Pomadasys corvinaeformis*** **(n = 52)** **IAi**	***Sphoeroides testudineus*** **(n = 19)** **IAi**
(1) Nematoda						
Non-identified taxon	0.000069					
(2) Cnidaria						
Non-identified taxon			0.000539			
Anthozoa	0.001729					
Total Cnidaria category						
(3) Mollusca						
Bivalvia	0.013236	0.000060	0.032070	0.000021	0.153315	0.777363
Gastrophoda	0.187773				0.000017	0.002023
Juvenil squid				0.008613		
Total Mollusca category	0.201009	0.000060	0.032070	0.008634	0.153332	0.779386
(4) Polychaeta						
Non-identified taxon			0.118038		0.025335	
(5) Crustacea						
Larva of Megalopa	0.006082		0.000404			
Zoea larval stage				0.000085		
Amphipoda	0.000086	0.000481		0.000382		
Isopoda			0.001970			
Copepoda	0.039743	0.000040		0.000020		
Cirripedia	0.037633	0.000001				
Brachyura		0.022107	0.039526	0.005299	0.041799	
Decapoda						
Lucifer sp.	0.013155			0.000559		
Tanaidacea	0.000173			0.000454		
Dendrobranchiata	0.368685	0.087716	0.657115	0.827719	0.467751	
Larva of *Puerulus* sp.				0.000168		
Rest of Decapoda	0.041161					
Rest of crustaceans	0.002998	0.001469		0.001935	0.002559	0.000070
Total Crustacean category	0.509717	0.111814	0.698015	0.836622	0.512109	0.000070
(6) Insecta						
Rest of insects (wings or legs)	0.001729				0.001706	
(7) Echinodermata						
Clypeasteroida					0.001109	0.204319
(8) Teleostei						
Otolith	0.000006					
Fry	0.005419	0.000196	0.001437	0.097101		
Larval stage of sea horse		0.000571				
Rest of Teleostei (scales and bones)	0.000138		0.004492	0.056970	0.171408	0.005911
Whole fish		0.876364				
Total Teleostei category	0.005563	0.877130	0.005929	0.154072	0.171408	0.005911
(9) Algae					0.001450	
(10) Vegetal matters	0.001314	0.000101				0.000141
(11) Animal matters	0.025631	0.010897	0.146784		0.069539	
(12) Sediment	0.252846			0.000692	0.064011	0.009753
(13) Rock	0.000017					
(14) Plastic matters						
Rigid plastic	0.000029					
Transparent soft plastic	0.000346					
Total Plastic category	0.000375					
(15) Non-identified items						0.000422
Total	1	1	1	1	1	1

Our results revealed that the species studied are considered carnivorous. *O. oglinum*, *L. breviceps*, *M. littoralis* and *P. corvinaeformis* show a tendency to carcinophagia; *C. nobilis* has tendency to piscivoria, and *S. testudineus* feeds mainly on bivalves.

2. *Feeding and reproductive activities*

According to precipitation data, the dry season occurred from August to December (rain mean = 0.14 mm), and the rainy season was from January to July (rain mean = 0.97 mm). The SRI analysis revealed that the species studied had higher feeding activity during the dry season; *C. nobilis* had highest SRI (2.46 ± 2.32), followed by *O. oglinum* (2.39 ± 1.27). On the other hand, the GSI was higher in the rainy season, and *S. testudineus* presented the highest GSI (3.04 ± 3.60) (Table 2).

3. *Histological features of digestive tube*

We described histological features in four species that represented each group.

O. oglinum: esophagus has concentric tissue layers of mucosa, submucosa, muscular, and serosa. We observed a transition from stratified epithelium (Figure 3a) to a simple columnar epithelium between esophagus and stomach. Stomach mucosa presents folds of the epithelium in the lamina propria forming the gastric pits. We observed numerous tubular gastric glands near the pylorus region (Figure 3b). Tunica muscular has two layers: external (longitudinal) and internal (circular) (Figure 3c). The pyloric region has strong muscular walls, with a developed muscular layer (Figure 3b). Intestine walls are supplied from mucosa, muscular, and serosa layers (Figure 3d). The epithelium contains cylindrical cells (for absorption) and mucosal cells with goblet-shape.

Table 2. Mean and Standard Deviation of the Stomach Repletion Index (SRI) and Gonadosomatic Index (GSI) of six fish species discarded by trawl in northeastern Brazil, from January to December 2012, according to rainy and dry seasons.

Species	**Rainy season**		**Dry season**	
	SRI	**GSI**	**SRI**	**GSI**
Opisthonema oglinum	2.06 ± 0.88	0.43 ± 0.65	2.39 ± 1.27	0.10 ± 0.05
Conodon nobilis	2.07 ± 2.21	0.26 ± 0.14	2.46 ± 2.32	0.09 ± 0.08
Menticirrhus littoralis	1.28 ± 0.55	0.67 ± 0.78	1.78 ± 1.61	0.21 ± 0.16
Larimus breviceps	0.74 ± 0.58	0.82 ±1.20	1.89 ± 2.29	0.59 ± 0.76
Pomadasys corvinaeformis	0.88 ± 0.63	0.73 ± 0.89	1.16 ± 1.02	0.27 ± 0.64
Sphoeroides testudineus	1.25 ± 2.77	3.04 ± 3.60	1.55 ± 1.13	1.88 ± 1.72

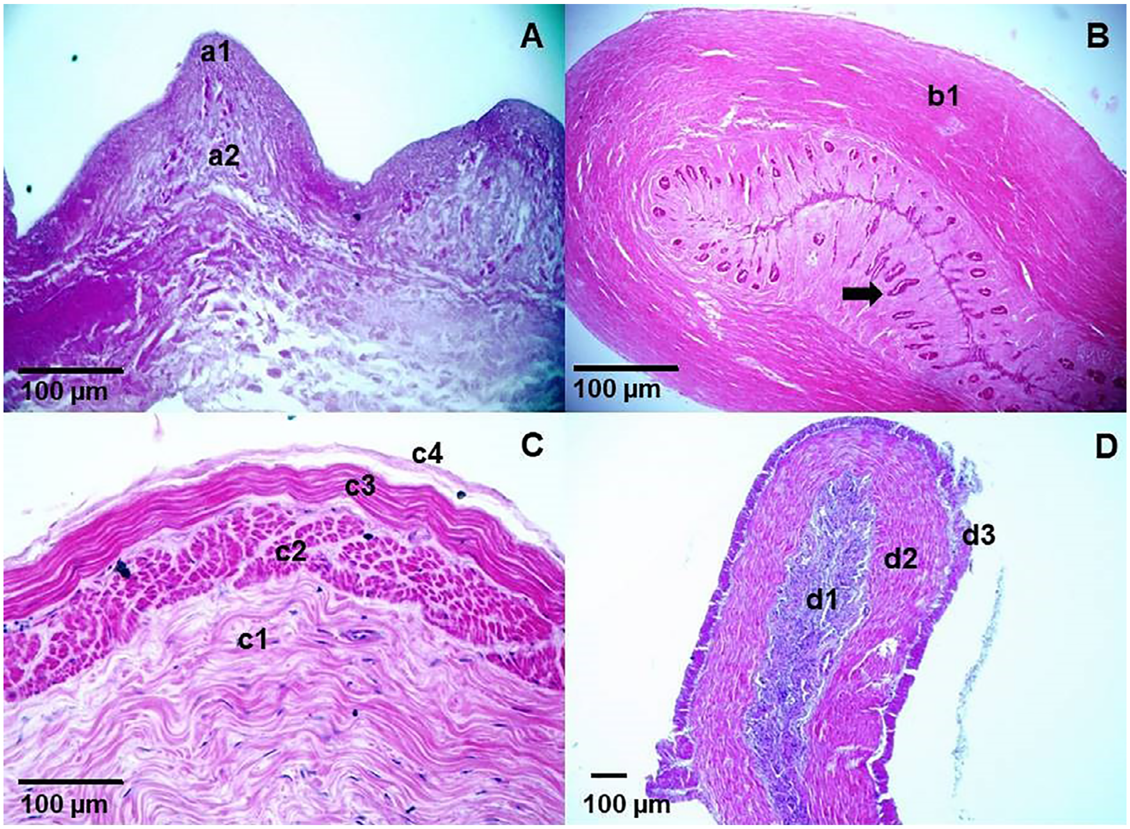

Figure 3. Histological section of digestive tube of *Opisthonema oglinum*. (A) Esophagus: stratified epithelium (a1) and lamina propria (a2). (B) Pyloric region of the stomach: muscular layer (b1) and tubular gastric glands (arrow). (C) Cardia region of stomach: lamina propria (c1); muscular layer: inner circular layer (c2) and outer longitudinal layer (c3); serosa layer (c4). (D) Small intestine: microvilli (d1); muscular layer (d2); serosa layer (d3). H.E. Bar 100 μm.

C. nobilis: esophagus with stratified epithelium (Figure 4a), and lamina propria formed by aglandular connective tissue. In the stomach and intestine, we observed a simple columnar epithelium. Stomach tunica muscular has an outer longitudinal and an inner circular layer, thinner than the layers in esophagus (Figure 4b). The intestine presents many villous structures (Figure 4c), thicker inner circular musculature, and serosa layer similar to the anterior portions of the digestive tube (Figure 4d).

L. breviceps: epithelium of digestive tube has features similar to those found in *C. nobilis.* We observed basophilic basal cells and mucus-secreting cells in the esophageal epithelium (not stained by hematoxilin-eosin) (Figure 5a). In the transition from esophagus to stomach, we noted a gradual increase in mucus-secreting cells. In the cardia region, walls were thick and reduced light, due to the large number of folds (Figure 5b), and tunica muscular with inner circular and outer longitudinal layers (Figure 5c). Gastric glands were found in the cecal mucosa from invaginations of the simple columnar epithelium (Figure 5d), and they secrete hydrochloric acid and pepsinogen. Intestine tunica muscular has thicker inner circular layer than outer longitudinal (Figure 5e). The pyloric caecum presented features similar to those in the intestine, but a thinner musculature (Figure 5f).

S. testudineus: the histological analysis revealed a pseudo-stomach characterized by less villi and thicker muscular tunica than intestine (Figure 6a, b, c, d), and absence of pyloric caeca. The division between the pseudo-stomach and intestine was evident only through microscopic examination.

Discussion

The relative abundance of *Opisthonema oglinum*, *Conodon nobilis*, *Larimus breviceps* and *Sphoeroides testudineus* was higher than the amount obtained by Feitosa et al. (2013) in the same site and considering bycatch. A previous study revealed that *Pomadasys corvinaeformis*, *Menticirrhus littoralis* and *L. breviceps* were negatively related to the fishing activity and the species were more vulnerable to shrimping trawl nets. On the other hand, *Pellona harroweri* individuals were captured after reaching the maturity size (Bomfim et al. 2019). Feitosa et al. (2013) also observed the impact of trawl nets to the species analyzed, and *C. nobilis* and *L. breviceps* were classified as less susceptible. *Pomadasys corvinaeformis* negatively related to fishing activity, despite the higher correlation with the survival criterion.

Sand beaches are considered feeding sites for several fish species, especially for the juvenile individuals (Suda et al. 2002). Our study site includes localities with high shrimp trawl activities, according to data from UNIDADE DE OPERAÇÃO DE EXPLORAÇÃO E PRODUÇÃO DO RIO GRANDE DO NORTE E CEARÁ – UO-RNCE (2012). Our results showed that shrimp had significant importance in the food composition of the fishes examined, once we found the penaeid post-larvae stage in stomach contents.

Diet composition of examined *O. oglinum* was based on Gastrophoda, Dendrobranchiata, Sediment and Cirripedia, with Crustacea as the main category. Vega-Cendejas et al. (1997) found that diet of *O. oglinum* in the Mexico Gulf was mainly composed of benthonic microcrustaceans and macrophyts. The most species classified in the order Clupeiformes are plankton-eating fishes, filtering water and retaining food items (Alves & Sawaya 1974, Nelson 2006).

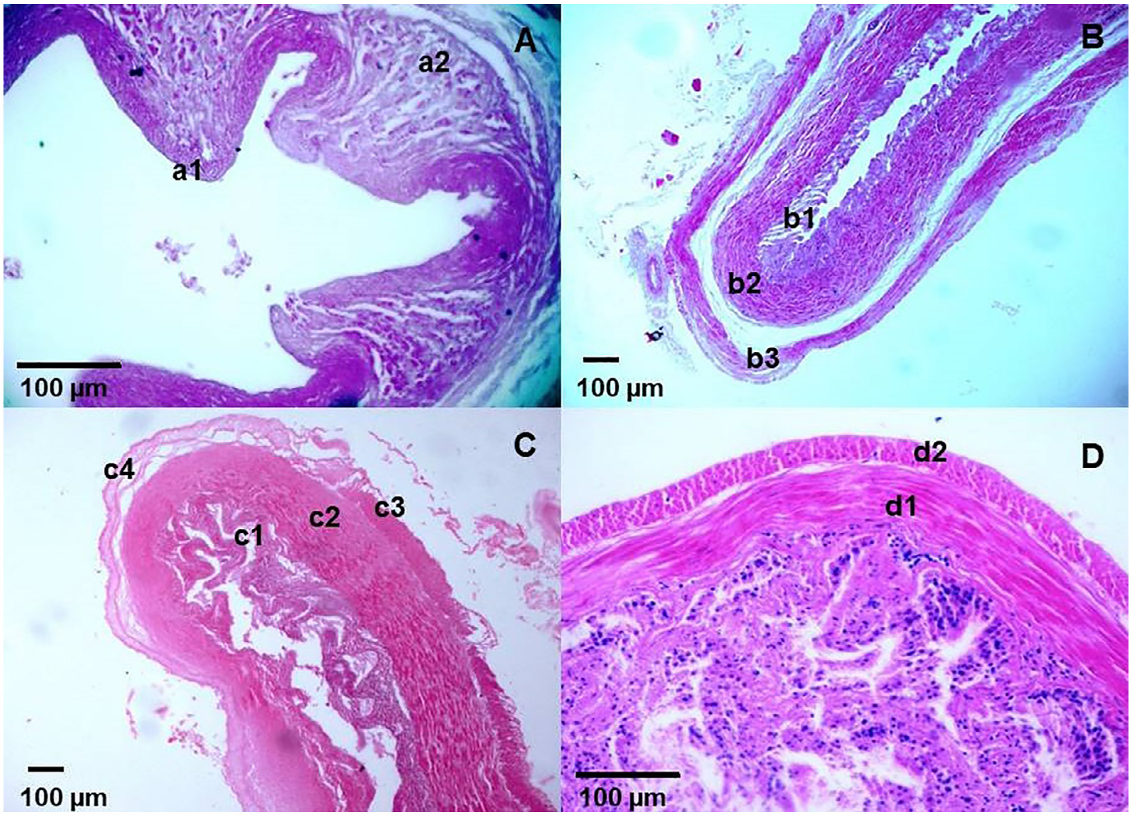

Figure 4. Histological section of digestive tube of *Conodon nobilis.* (A) Esophagus: stratified epithelium (a1) and lamina propria (a2). (B) Pyloric region of the stomach: folds in the mucosa layer (b1); muscular layer – inner circular layer (b2) and outer longitudinal layer (b3). (C) Small intestine: microvilli (c1); muscular layer – inner circular layer (c2) and outer longitudinal muscle layer (c3); serosa layer (c4). (D) Pyloric caecum: muscular layer – inner circular layer (d1) and outer longitudinal layer (d2). H.E. Bar 100 µm.

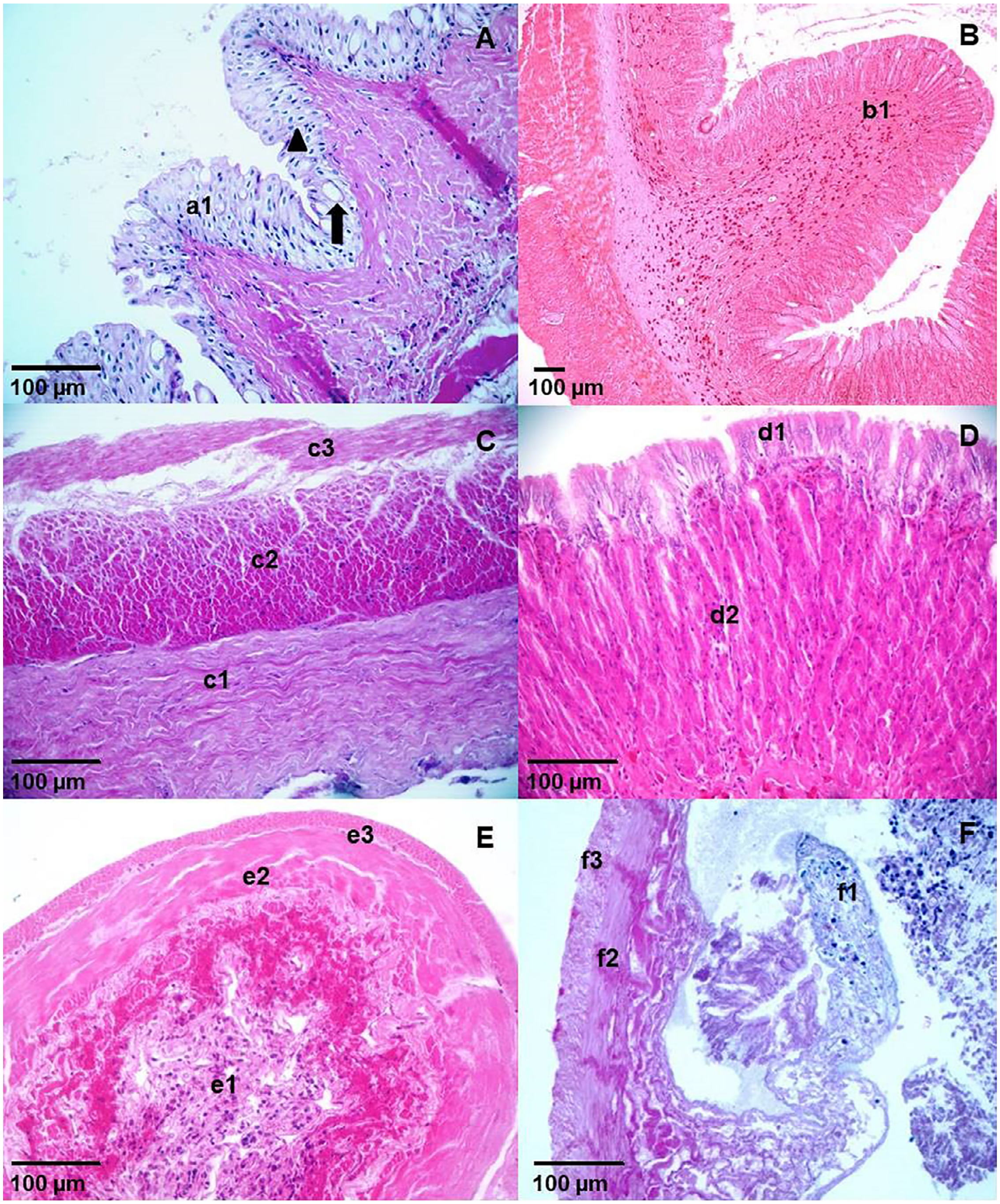

Figure 5. Histological section of digestive tube of *Larimus breviceps.* (A) Esophagus: simple columnar epithelium (a1), basophilic basal cells (arrowhead) and mucus-secreting cells basal cells (arrow). (B) Cardia region of the stomach: folds (b1). (C) muscular layer – inner circular layer (c1) and outer longitudinal layer (c2); and vascularized serosa layer (c3). (D) Cecal mucosa of the stomach: simple columnar epithelium (d1) and gastric glands (d2). (E) Small intestine: microvilli (e1); muscular layer – inner circular layer (e2) and outer longitudinal layer (e3). (F) Pyloric caecum: microvilli (f1); muscular layer – inner circular layer (f2) and outer longitudinal muscle layer (f3). H.E. Bar 100 µm.

However, *O. oglinum* seems to be an opportunistic species presenting trophic adaptability according to most food items available in different seasons and types of marine habitat (Wootton 1990). The opportunistic feeding behavior was already described for many fish species in estuaries (Elliot et al. 2007).

In our study site, *C. nobilis* has tendency to piscivory and we identified 13 food items in the stomach contents, as described previously in a study carried out in Jaguaribe beach, Brazil, which reported Decapoda as the main food category (Lopes & Oliveira-Silva 1999). We considered Teleostei scales as accidental ingestion without indicating a lepidophagic habit.

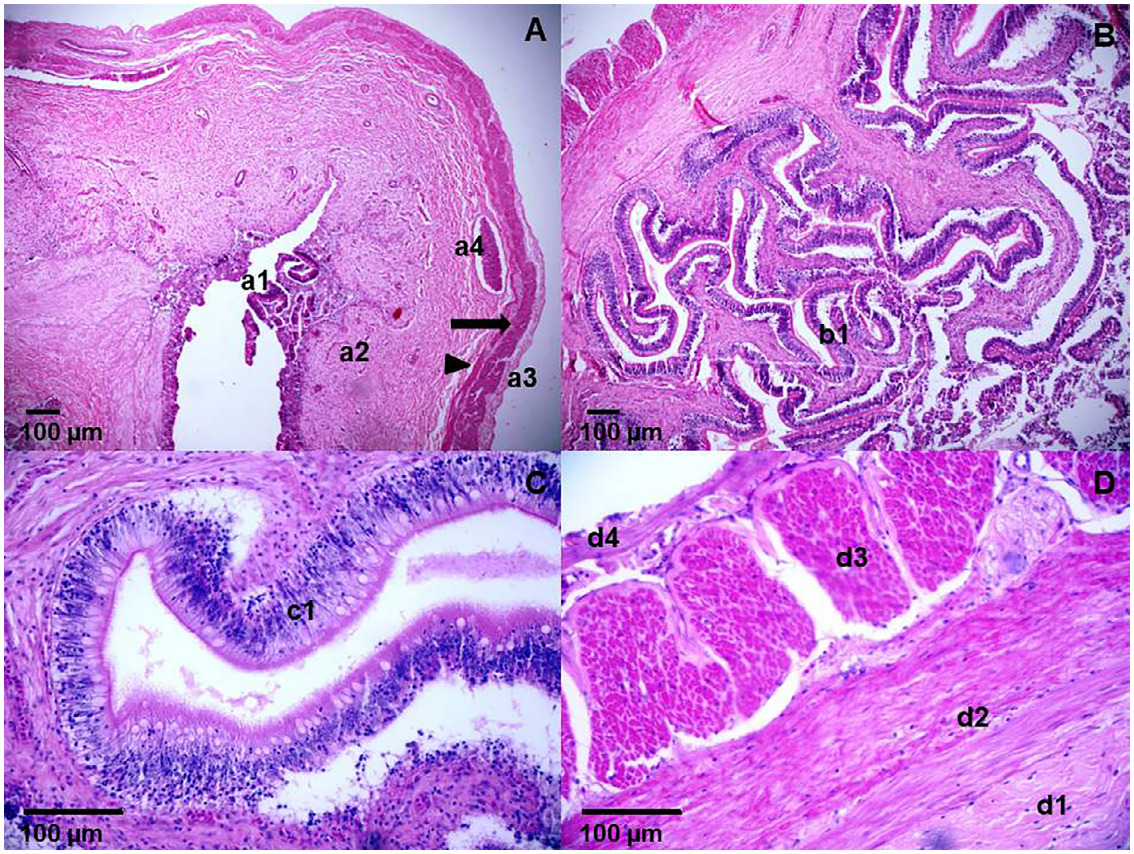

Figure 6. Histological section of digestive tube of *Sphoeroides testudineus*. (A) Pseudo-stomach: microvilli (a1); muscular layer of mucosa (a2) – inner circular layer (arrowhead) and outer longitudinal layer (arrow); serosa layer (a3); blood vessel (a4). (B, C and D) Small intestine: microvilli (b1); simple cylindrical epithelium (c1); submucosa layer (d1); muscular layer of mucosa – inner circular layer (d2) and outer circular layer (d3); serosa layer (d4). H.E. Bar 100 µm.

Menticirrhus littoralis ate mainly Dendrobranchiata, followed by Polychaeta. Similar findings were described by previous study in southeastern Brazil with Amphipoda, Polychaeta and Dendrobranchiata presenting high occurrence frequency (Rodrigues & Vieira 2010).

Our analysis revealed that *L. breviceps* feeds mainly on crustaceans in the study site with Sediment considered as accidental ingestion, corroborating previous studies in different Brazilian areas (Ponta da Ilha beach - Bahia State, and Jaguaribe beach - Pernambuco State) (Moraes et al. 2004, Lopes & Oliveira-Silva 1999). Moraes et al. (2004) indicated algae also as accidental ingestion; however, we did not find this type of food item in our samples.

Our results showed that Crustacean (represented by Brachyura, Dendrobranchiata and rest of crustaceans) was the main food category of *P. corvinaeformis* examined. In Fortaleza, Ceará State – Brazil, the diet composition analysis of this species revealed, in order of importance, crustaceans, polychaetes, fish and algae. In southeastern Brazil, crustaceans comprised five of the nine food categories (non-identified crustacean fragments, zoea larvae, amphods, copepods and shrimp) (Costa et al. 1995, Denadai et al. 2013).

In the Potiguar basin, diet of *S. testudineus* was mainly composed by bivalves and echinoderms (order Clypeasteroida). A previous study reported bivalves (especially in the juvenile life-stage), Ceratopogonidae larvae, calanoids, gasthropods, ostracods, cyclopoids, decapods, and barnacles as food items (Araújo et al. 2016). The species studied were considered carnivorous and their diet composition, based on benthic, demersal and pelagic animals, can influence their position in the water column as stated by Wootton (1989). Therefore, these fish species may become more susceptible trawl catches.

The Stomach Repletion Index (SRI) varied between the dry and rainy seasons. The dry season presented higher feeding activity (August-December). Seasonal variation can provide information on life cycle of preys or food competition (Zavala-Camin 1996). In our study site, strong winds occurred between September and December, decreasing shrimp trawls activity on the coast, and shrimp may be more available for the fish species examined. In northeastern Brazil, the breeding time for fishes occurs in the rainy season (January-July) (Bennemann et al. 1996), period with higher gonadosomatic index (GSI) and lower SRI for the fish species investigated. During the rainy season, gonads increase, resulting in a mechanical pressure on the digestive tube and a food ingestion decrease (Bennemann et al. 1996, Costa et al. 2005), explaining our results. Furthermore, higher SRI in the dry season suggests that fishes can try to save energy for the breeding time (rainy season).

Morphology of digestive tube can affect the feeding habit and diet plasticity (Gosavi et al. 2019), and our findings confirm the relation between diet composition and digestive tube morphology, considering the histological features (e.g. pyloric caeca and stomach with thick muscular layer) of the fish species examined. The stomach has a varied shape and structure depending on food habits of fishes, and may be absent in some species (Canan et al. 2012). Examined *C. nobilis* and *L. breviceps* presented similar structures with other carnivorous fishes, such as well-developed pyloric caeca, short intestine and muscular stomach (Alabssawy et al. 2019). We found mucus-secreting cells in the esophageal epithelium of *L. breviceps*, and in the transition from esophagus to stomach, we noted a gradual increase in this type of cells. The mucus hydrates the food and favors its flow up to intestine, protecting the mucosa against mechanical injuries (Silva et al. 2018), which could be caused by ingestion of whole food items, as observed in our analysis of the diet composition. In the samples from *S. testudineus,* we observed a pseudo-stomach with thick muscular layer, probably involved with mechanical digestion of mollusk and echinoderms (food items found during our analysis); however, the division between the esophagus, stomach, and intestine was not evident through macroscopic examination. As previously reported by Alves & Sawaya (1974), we observed well-developed longitudinal and circular tunica in the muscular layer of pyloric region of *O. oglinum* samples, an important feature considering accidental sediment ingestion during capture of benthic animals.

Our Study confirms that the digestive tube of the examined species has morphological adequacy for carnivorous feeding habit, and provides useful information on tissue structures associated to diet composition, which is sometimes insufficiently answered through macroscopical description.

Acknowledgments

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Author contributions

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Conflicts of interest

The authors declare that they have no conflict of interest related to the publication of this manuscript.

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Article

Description of mating behavior, life cycle, and antennal sensilla of *Cyclocephala putrida* Burmeister, 1847 (Coleoptera, Scarabaeidae, Dynastinae)

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Abstract: The genus *Cyclocephala* is common in Brazil (Coleoptera, Scarabaeidae, Dynastinae). The adults of some species are important pollinators, and the larvae develop in the soil, feed on organic matter, and contribute to nutrient cycle, but immatures of some species feed on plant roots, and some were registered causing damage in crops. The mating process of some phytophagous scarab beetles has a chemical recognition step, and the antenna is the main structure involved in the detection of odorants associated with insect communication. In the present study the mating behavior, life cycle, and antennal sensilla of *C. putrida* are described. The study was conducted at the Universidade Estadual de Mato Grosso do Sul, Cassilândia, Brazil. Adults were collected by a light trap installed from January 2016 to December 2017 and were taken to the laboratory for studies. Adults swarms are brief and were registered from January to February, and specimens were mostly collected at 20:00 to 22:00h. Chemical recognition may occur at least during one of the mating steps, during which the couple kept their antennae moving and the lamellae open, while females select males. In laboratory, the mating process lasted 7.5 minutes on average. The antennae of females have about 3399 sensilla and males about 4229 sensilla. Sensilla placodea types I, II, and III are the most abundant, and sensilla ampullacea, basiconica, and coeloconica are also present. The embryonic period lasted 16.0 days; first, second and third instars lasted 16.0, 48.3, and 165.3 days, respectively. The pupal period lasted 24.0 days. The period between egg deposition and adult emergency is about 271.5 days.

Keywords: *Chemical Communication, Chemical Receptors, Cyclocephalini, Neotropical, Swarming Flights.*

Descrição do comportamento de cópula, ciclo de vida e sensilas antenais de *Cyclocephala putrida* Burmeister, 1847 (Coleoptera, Scarabaeidae, Dynastinae)

Resumo: O gênero *Cyclocephala* é comum no Brasil (Coleoptera, Scarabaeidae, Dynastinae). Os adultos de algumas espécies são importantes polinizadores, e a larva desenvolve-se no solo, alimenta-se de matéria orgânica e contribui para a ciclagem de nutrientes, mas imaturos de algumas espécies alimentam-se de raízes de plantas, e alguns são registrados causando danos em plantas cultivadas. O processo de cópula de algumas espécies de Scarabaeidae fitófagos, apresentam reconhecimento químico, e nas antenas aparecem várias estruturas responsáveis pela detecção dos odores envolvidos na comunicação. No presente trabalho o comportamento de cópula, ciclo de vida e sensilas antenais de *Cyclocephala putrida* são descritos. O estudo foi conduzido na Universidade Estadual de Mato Grosso do Sul, Cassilândia, Brasil. Adultos foram coletados com armadilha luminosa instalada de Janeiro de 2016 a Dezembro de 2017, e levados para laboratório para estudos. Os adultos revoam por curto período de janeiro a fevereiro, e os espécimes foram coletados em maior quantidade das 20:00 às 22:00 h. O reconhecimento químico possivelmente ocorre durante as etapas que envolvem o comportamento de cópula, no qual o casal mantém as antenas em movimento e as lamelas abertas e as fêmeas selecionam os machos. Em laboratório a cópula dura 7,5 minutos em média. As antenas das fêmeas possuem 3399 sensilas e os machos 4229 sensilas, e as sensilas placódeas dos tipos I, II e III, foram as mais abundantes e sensilas ampuláceas, basicônicas e coelocônicas também estão presentes. O estágio de ovo durou 16,0 dias; o primeiro, segundo e terceiro instar duraram 16,0, 48,3 e 165,3 dias, respectivamente. O período pupal durou 24,0 dias. O período entre deposição dos ovos e emergência dos adultos é de 271,5 dias em média.

Palavras-chave: *Comunicação Química, Receptores Químicos, Cyclocephalini, Neotropical, Período De Voo.*

Introduction

The genus *Cyclocephala* Dejean, 1821 (Coleoptera, Scarabaeidae, Dynastinae, Cyclocephalini) is distributed through the Americas, and about 123 species have been recorded in Brazil (Grossi & Vaz-de-Mello 2019).

Several adults were registered as flower visitors, with potential to be pollinators, and the visited plants are usually used as feeding and mating sites (Moore & Jameson 2013). Adults of *Cyclocephala forsteri* Endrödi, 1963 have been associated with flowers of *Acrocomia aculeata* Lodd. ex Mart. ("macaúba" Arecaceae) (Oliveira & Ávila 2011), while adults of *Cyclocephala melanocephala* (Fabricius, 1775) have been found in flowers of *Cereus peruvianus* (L.) Mill. (Cactaceae) (Silva & Sazima 1995), sunflowers ("girassol", *Helianthus annuus* L., Asteraceae) (Camargo & Amabile 2001), and passion-fruit flowers ("maracujazeiro", *Passiflora edulis* Sims, Passifloraceae) (Dias & Rodrigues 2018). *Cyclocephala literata* Burmeister, 1847 consumes flowers of *Magnolia ovata* Spreng. (Magnoliaceae) and uses the plant as a mating site (Gottsberger et al. 2012). *Cyclocephala celata* Dechambre, 1980 and *Cyclocephala cearae* Höhne, 1923 have been associated with *Taccarum ulei* Engl. & K. Krause (Araceae) plants and their adults were observed feeding and copulating on its flowers (Maia et al. 2013). It is known that some phytophagous scarab beetles locate plants by detecting volatile odorants using the sensilla on their antennae (*Anomala cuprea* Motschulsky, 1854; Larsson et al. 2001), and the same may occur for Cyclocephalini beetles (Barbosa & Rodrigues 2016; Maia et al. 2013).

Some Scarabaeidae species release sexual pheromones to chemical recognition, and females select males before copulation (e.g. Facundo et al. 1999, Rodrigues et al. 2014). As well as plant volatiles, insect structures involved in detecting pheromones are antennal sensilla, which are mainly distributed on the surface of lamellae (e.g. Leal & Mochizuki 1993, Kim & Leal 2000).

Before copulation, females return to the soil for egg deposition, and their biological cycle varies with species. The time between egg deposition and adult emergence is about 229 days for *C. verticalis* Burmeister, 1847 (Rodrigues et al. 2010), about 164 days for *C. celata* (Souza et al. 2014), and about 108 days for *C. distincta* Burmeister, 1847 (Souza et al. 2015).

The larvae of *Cyclocephala* use soil with organic matter as food, therefore being important for nutrient cycling such as *C. paraguayensis* Arrow, 1913 (Albuquerque et al. 2014). On the other hand, these larvae may consume plant roots, and some species can cause damage to crops such as *C. forsteri* which has been reported as potential pest to soybean crops ("soja", *Glycine max* (L.) Merr, Fabaceae) (Santos & Ávila 2007), or such as *Cyclocephala flavipennis* Arrow, 1914, which has been registered damaging pastures (Duchini et al. 2017).

Although studies have been conducted on *Cyclocephala putrida* Burmeister, 1847 biology in Cassilândia, state of Mato Grosso do Sul (Brazil), little information is known for this species. Cherman et al. (2014) found larvae of the species in cultivated and non-cultivated areas in the state of Rio Grande do Sul (Brazil), and Bonivardo et al. (2015) reported immatures associated with pasture and maize ("milho", *Zea mays* L., Poaceae) in Argentine.

Regarding the above-mentioned biological and economic importance of the genus *Cyclocephala*, the present work aims to describe the mating behavior, the antennal sensilla, and life cycle of *C. putrida*.

Material and Methods

The study was conducted at the Universidade Estadual de Mato Grosso do Sul (UEMS), Campus of Cassilândia, MS (19° 06' 48" S; 51° 44' 03" W). Sampling and rearing procedures, as well as analysis strategies, were modifications of Rodrigues et al. (2008, 2010).

Adults of *C. putrida* were collected using a light trap model "Luiz de Queiroz" from January 2016 to December 2017. The trap was installed in a pasture area (*Urochloa decumbens* Stapf cv Basilisk, Poaceae) and was turned on daily at 18:00 and turned off at 6:00 of the next day. The insects were collected every 60 minutes, and collected adults were sexed by observing male dilated protarsomeres (Figure 1), and couples were placed in 500 mL plastic containers with half-filled with the same soil from where adults were collected. The adults were reared with slices of bananas (fruit of *Musa* sp., Musaceae) as food source (see Ferreira et al. 2016). Each couple was observed from February 2016 to February 2017 to behavior observations.

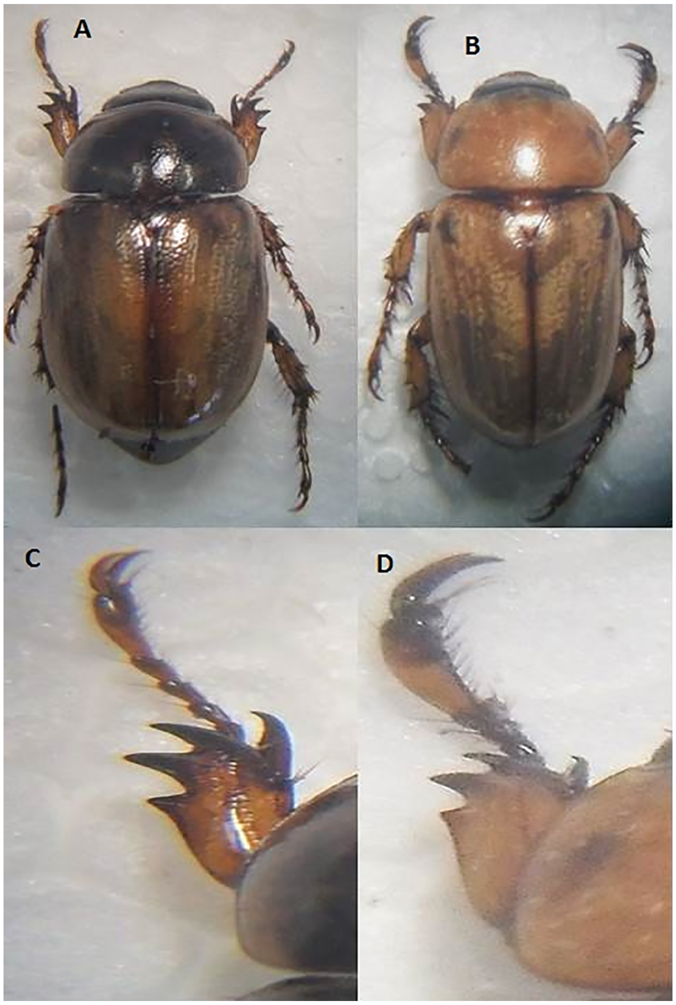

Figure 1. *Cyclocephala putrida* Burmeister,1847. A-B) male and female habitus, dorsal; C – D) male and female protarsus.

The containers were checked weekly when the soil was sieved to egg separation. After removed, the eggs were individualized and placed in other 250 mL plastic containers one-third filled whit soil, which were maintained in a climatized chamber (26 ± 1 ºC and 12 hours scotophase).

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Each newly hatched larva was transferred to a 250 mL plastic container with soil and *U. decumbens* seedlings. Cranium width and body total length were weekly measured (Figure 2).

Flight activity was observed from February 2 to 27, and data were transformed into $\sqrt{x+1}$ and submitted to analysis of variance (ANOVA). Means were grouped and compared by the Scott-Knott test ($p < 0.05$) using SISVAR software. Data on average temperature (◦C), precipitation (mm), and solar radiation (kJ/m^2) in Cassilândia, were obtained from the Instituto National de Meteorologia (INMET).

The study of antennal sensilla was based on antennal clava of 10 dissected specimens. Antennal segments were formerly treated in a 10% potassium hydroxide solution at 80°C for 60 minutes. The pieces were then successively washed in distilled water, 70% ethanol, 80% ethanol, and 90% ethanol, respectively (Romero-Lópes et al. 2010). Images were taken by a scanning electron microscope (model JSM 5410) at the Laboratório de Microscopia Eletrônica of the Universidade Estadual Paulista, campus of Jaboticabal, state of São Paulo, Brazil. Part of dissected lamellae was mounted on slides with Hoyer's solution (Johnson & Triplehorn 2005) and observed under a Nikon microscope model E2000. Sensilla terminology follows Keil (1999). Meinecke (1975) was used as an alternative sensilla terminology (in brackets) to easily comparison of terms. The insects were deposited in the UEMS entomological collection, campus of Cassilândia.

Results

Mating behavior. Four hundred and fourteen (414) adults were collected in 2016, 132 in January and 282 in February, which indicates a short swarming period. From 18:00 to 19:00 h the brightness decreased from 131.8 KJ/m² to 0, beetles flew from dusk till dawn (from 18:00 to 6:00 of the next day), and swarm activity was high at nightfall (from 20:00 to 22:00 h). Temperature ranged from 28.5 ºC to 23 ºC during the flight (from 18:00 to 6:00 of the next day; Figure 3).

Figure 2. *Cyclocephala putrida* Burmeister, 1847. A) Eggs, B) Larvae in containers with soil and seedlings of *Urochloa decumbens*. C) measurement of larvae.

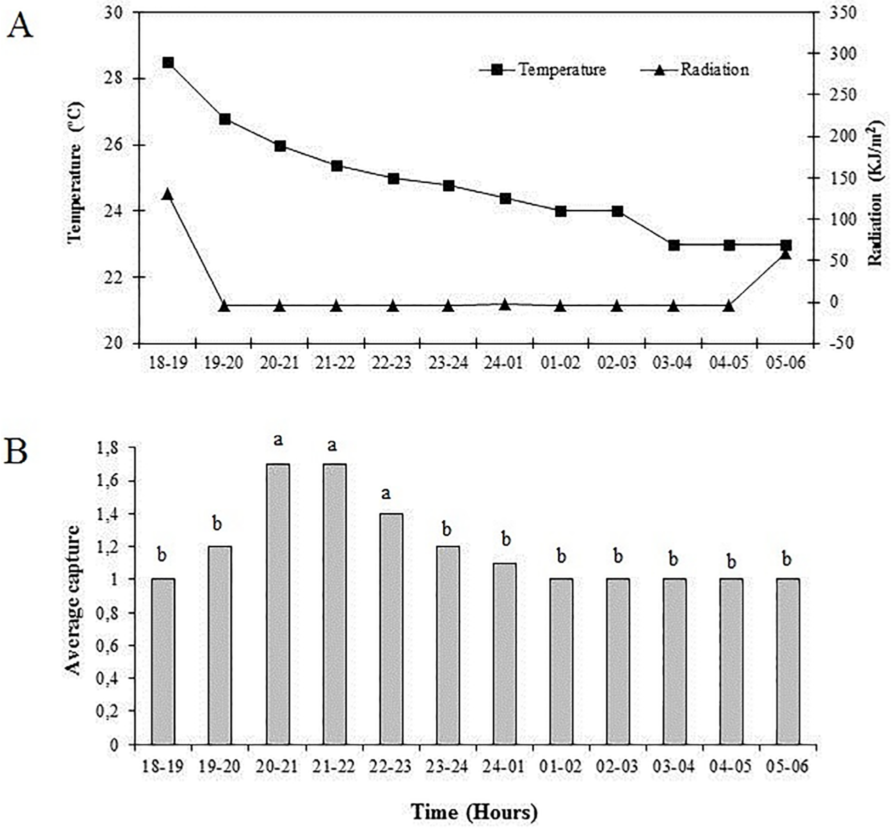

Figure 3. Radiation (kJ/m^2) and average temperature (ºC) obtained from the weather station (INMET) in Cassilândia, MS, Brazil, from 2 to 27 February 2016. A). Adults of *Cyclocephala putrida* collected with a light trap in the same period. B) Bars with the same letter are not statistically different (P<0.05, Scott-Knott test).

Adults remained buried in the soil during the day and started flying from 18:00 onwards. After leaving the soil, the beetles flew for 12.6 ± 1.86 min (11-15) when landed on plants for feeding and mating. Then, they started walking while their antennae kept moving and lamellae remained open. Afterwards, some couples were formed (Figure 4) and mated, with antennae movement being maintained during all stages.

Six of the 30 couples formed mated. At the first stage, males walked over females from behind (n=5) or side (n=1) and repeatedly touched female pygidium or apex of elytra with their antennae and protarsi. After, males climbed on females dorsum (n = 6) using their protarsal claws to seize females, remaining in such position for 0.55 ± 0.22 min (0.39-1.0). Sometimes, female refused the male for mating (n=4) and walked away from it (Figure 4). If accepted by the female (n=6), the male griped the female elytra, using all six legs. Then, rhythmical movements were observed in the male abdomen while its aedeagus was exposed, and the copulation began.

The copula lasted 7.5 ± 1.87 min (6-10) and occurred from 00:00 to 01:00 h. During the process, females stood still (n=6) and copulate only once. After copulation, males climbed off females and retracted their aedeagus, and then the pair quickly separated from each other. This final step lasted 7 ± 0.31 sec (5-12).

Antennal sensilla (Table 1). The entire outer surface of the antennal clava is covered by sensilla chaetica (long and short setae) and sensilla trichodea (minute setae, Figure 5), which were identified mainly as tactile (mechanoreceptors) but also as gustatory (contact chemoreceptors) sensilla (Keil 1999). Whereas the inner surface of clava (the lamellar side in contact with another lamella when the clava is close) was observed covered by sensilla ampullacea, basiconica, coeloconica, and placodea.

Sensilla ampullacea were identified as pores on the surface of the lamella. In Hymenoptera (Insecta), antennal pores were identified as CO_2 receptors (Keil 1999); therefore, their function in scarab beetle need to be checked. Sensilla basiconica were identified as type I (Meinecke 1975: L4) and resembles minute setae enclosed in a depressed pit, with the apex projected over pit limits. Two types of sensilla coeloconica were observed, and both are ovoid and enclosed into a depressed pit, the type I (Meinecke 1975: L1) had a pointed apex and the type II (Meinecke 1975: L2) had blunt apex.

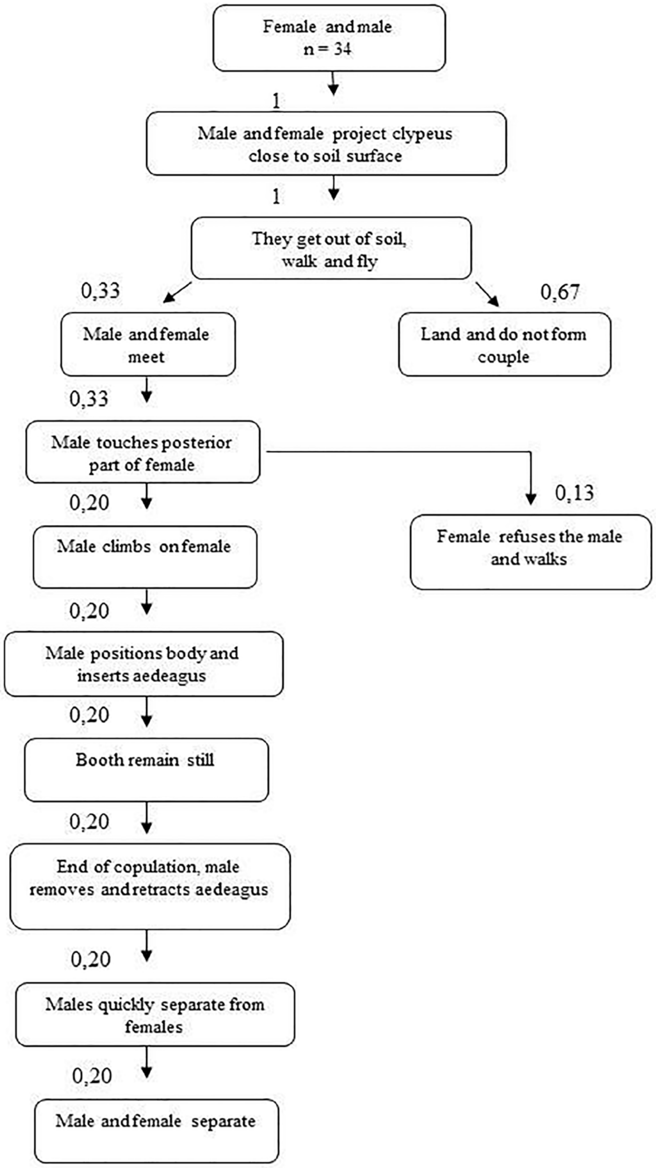

Figure 4. Ethogram of the mating behaviour of *Cyclocephala putrida*, Burmeister, 1847 in the laboratory.

Table 1. Sensilla of antennal lamella of *Cyclocepha putrida*.

Sensillum	proximal lamella		medial lamella		Distal lamella		Total
	external (proximal)	internal (distal)	external (distal)	internal (proximal)	external (distal)	internal (proximal)	
Female							
Placodea	0	943	645	833	0	663	3084
Coeloconica	0	100	78	59	0	52	289
Basiconica	0	11	6	6	0	3	26
Total	0	1054	729	898	0	718	3399
Males							
Placodea	0	1004	960	986	0	1066	4016
Coeloconica	0	66	33	11	0	80	190
Basiconica	0	7	4	4	0	8	23
Total	0	1077	997	1001	0	1154	4229

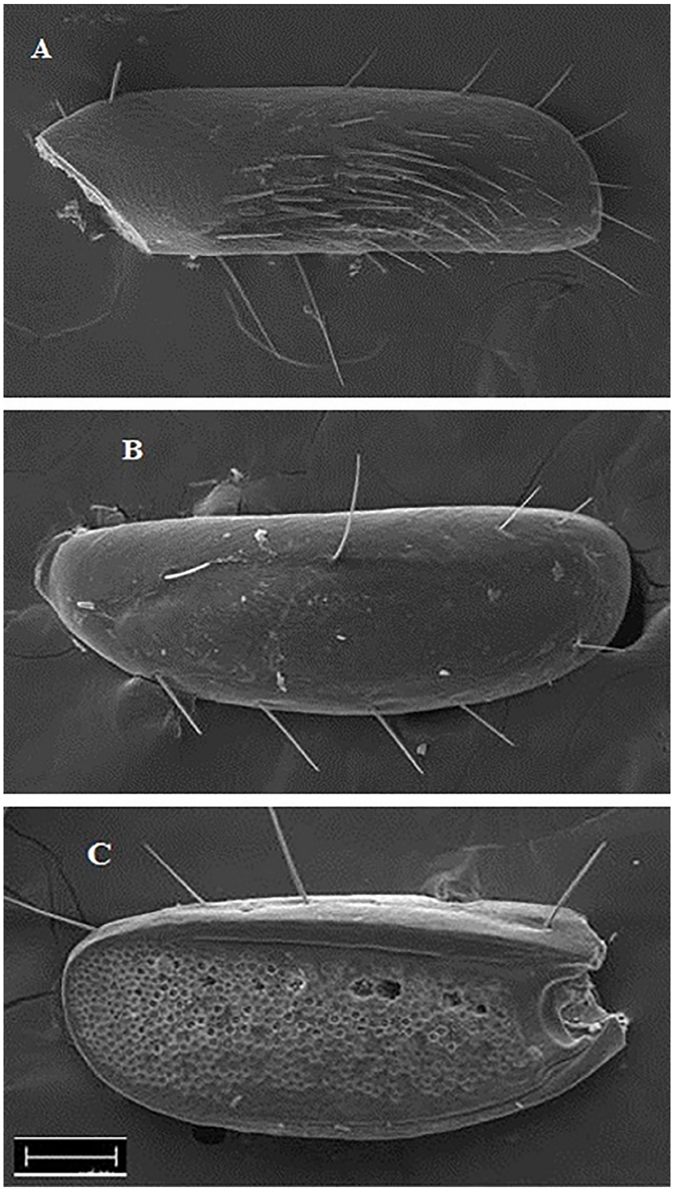

Figure 5. *Cyclocephala putrida*, Burmeister, 1847, antennae. A) proximal lamella, external (proximal side), B) distal lamella, external (distal side), C) medial lamella, external (distal side). Scale: 100 micrometers.

Three types of sensilla placodea are present: type I (Meinecke 1975: G2) is a plate surrounded by a ditch, the surface is irregularly reticulate and has mean diameter of 7.01 mμ (3.3-10.0); type II (Meinecke 1975: G1) is similar to type I but with a smooth surface and mean diameter of 6.18 mμ (3.89-10.0); type III (Meinecke 1975: J4) is almost smooth and the peripherical ditch is absent, mean diameter of 9.64 mμ (8.33-10.0) (Figure 6).

Females have a total of 3399 sensilla in lamellae, of which 3084 (90.7%) are sensilla placodea, 289 (8.5%) are sensilla coeloconica, and 26 (0.8%) are sensilla basiconica. Males have a total of 4229 sensilla, of which 4016 (95.0%) are sensilla placodea, 190 (4.5%) sensilla coeloconica, and 23 (0.5%) sensilla basiconica. Both sexes have a sparse amount of sensilla ampullacea in inner clava surface.

Life cycle. In the laboratory, 22 eggs were obtained in February and 14 eggs in March. The eggs showed 100% viability (Table 2). The mean duration of egg stage was 16.0 days, first-instar larvae 16.0 days, second-instar larvae 48.3 days, third-instar larvae 165.3 days, and pupae 24 days. The first and second instars showed 80.5 and 60% viability, respectively. The initial development stages had lower viability, as they are the most sensitive during the larval phase. The mean period between egg deposition and adult emergence was 271.5 days, and viability was 41.6%. Cranium width of first-instar larvae was 1.3 mm, of second-instar larvae was 2.4 mm, and of third-instar was 4.17 mm. The body length of first-instar larvae was 4.4 mm, of second-instar larvae was 7 mm, and of third-instar larvae was 15.91 mm. The third-instar larvae had a light grey colour that changed to white when pre-pupal stages started. Pre-pupae stop eating and make pupa chamber, possibly with aid of saliva or moist soil, manipulated with mouth appendages.

Discussion

Adults of *C. putrida* occurred in the field from January to February, as with *C. paraguayensis* found by Riehs (2006) in South of Brazil, but different from *C. melanocephala*, *C. tucumana*, *C. verticalis*, and others, which occur at beginning of rainy season, in September and October (Rodrigues et al. 2010, Nogueira et al. 2013). The occurrence patterns of some *Cyclocephala* species may be a strategy to avoid competition, but this hypothesis needs to be checked further.

Field observation showed that *C. putrida* start to flight at dusk, as other *Cyclocephala* species, as *C. verticalis* (Rodrigues et al. 2010, Barbosa & Rodrigues 2016), and other phytophagous Scarabaeidae, as *Anomala testaceipennis* Burmeister, 1856 (Rutelinae) (Rodrigues et al. 2014).

In the laboratory, adults started swarming at 00:00 h and copula started at 1:00 h. Adults of *C. verticalis*, when coupled in the laboratory, started copulating regardless of the day (Rodrigues et al. 2010).

Mating of *C. putrida* encompassed several stages, including coming out of the soil, start walking, swarming, forming couples, selecting females, and copulating. This process is quite similar to that observed in other *Cyclocephala* species such as *C. melanocephala*, *C. testaceipennis*, and *C. verticalis* (Rodrigues et al. 2014, 2018, Barbosa & Rodrigues 2016). Female selection in Scarabaeidae may be related to the recognition of male sexual maturity as in *Canthon cyanellus cyanellus* (LeConte, 1859) (Fávila 1988) and *C. verticalis* (Barbosa & Rodrigues 2016), and is might be intermediated by chemical communication (see Rodrigues et al. 2019).

Table 2. Stages and instar development to *Cyclocephala putrida*.

stage or instar	mean duration (days)	variation (days)	number of repetitions	viability (percent)
egg	16.0 + 1.03	15-18	36	100
first-instar larva	16.0 + 1.03	15-18	29	80.5
second-instar larva	48.3 + 3.56	45-54	18	62
third-instar larva	165.3 + 4.81	150-180	18	100
pupae	24.0 + 1.56	20-28	16	88.9
egg to adult	271.5 + 19.28	245-298	15	41.6

At the beginning of mating behavior, adults showed active movements of antennae and lamellae, probably for detection of pheromones released by other specimens. Some studies with Scarabaeidae have found that lamellar sensilla are involved in the detection of sexual pheromones, and these sensilla are more abundant in males than in females (Romero-López et al. 2004, Tanaka et al. 2006, Mutis et al. 2014). Also, males of *C. putrida* had more sensilla than females. Sensilla placodea was predominant in males and females of this species, while sensilla coeloconica and sensilla basiconica occurred in smaller quantities. Such dominance of sensilla placodea was also found in other Dynastinae species such as *Oryctes rhinoceros* (Linnaeus, 1758) (Renou et al. 1998), and in other phytophagous subfamilies such as *Anomala iconstans* Burmeister, 1844 (Rutelinae, Rodrigues et al. 2019). Larsson et al. (2001) studied the antennal sensilla of *Anomala cuprea* (Hope, 1839) and concluded that the outer sensilla placodea are responsible to detecting sexual pheromones while the inner ones are responsible for detecting plant volatiles.

The predominance of sensilla placodea may be related to important roles in detection food resources and adult info-chemicals (sexual or aggregative).

Concerning the life cycle of *C. putrida*, the period between egg deposition and adult emergence was on average of 271.5 days, so it could be considered a long life beetle if compared to other *Cyclocephala* species. Long cycles were also noticed for *C. melanocephala* (113 days) (Nogueira et al. 2013), *C. paraguayensis* (171 days) (Albuquerque et al. 2014), *C. tucumana* (211.2 days) (Nogueira et al. 2013), *C. verticalis* (228.6 days) (Rodrigues et al. 2010) (Table 3). The embryonic phase of *C. putrida* had a similar length to that other *Cyclocephala* species; however, the first instar lasted 16 days, a period shorter than in the other species (Table 3). The second and third instars of *C. putrida* lasted, in days, shorter than did *C. tucumana*, which lasted 66.1 and 176.8 days, respectively (Table 3). And its pupal phase was also shorter than *C. celata* (Table 3).

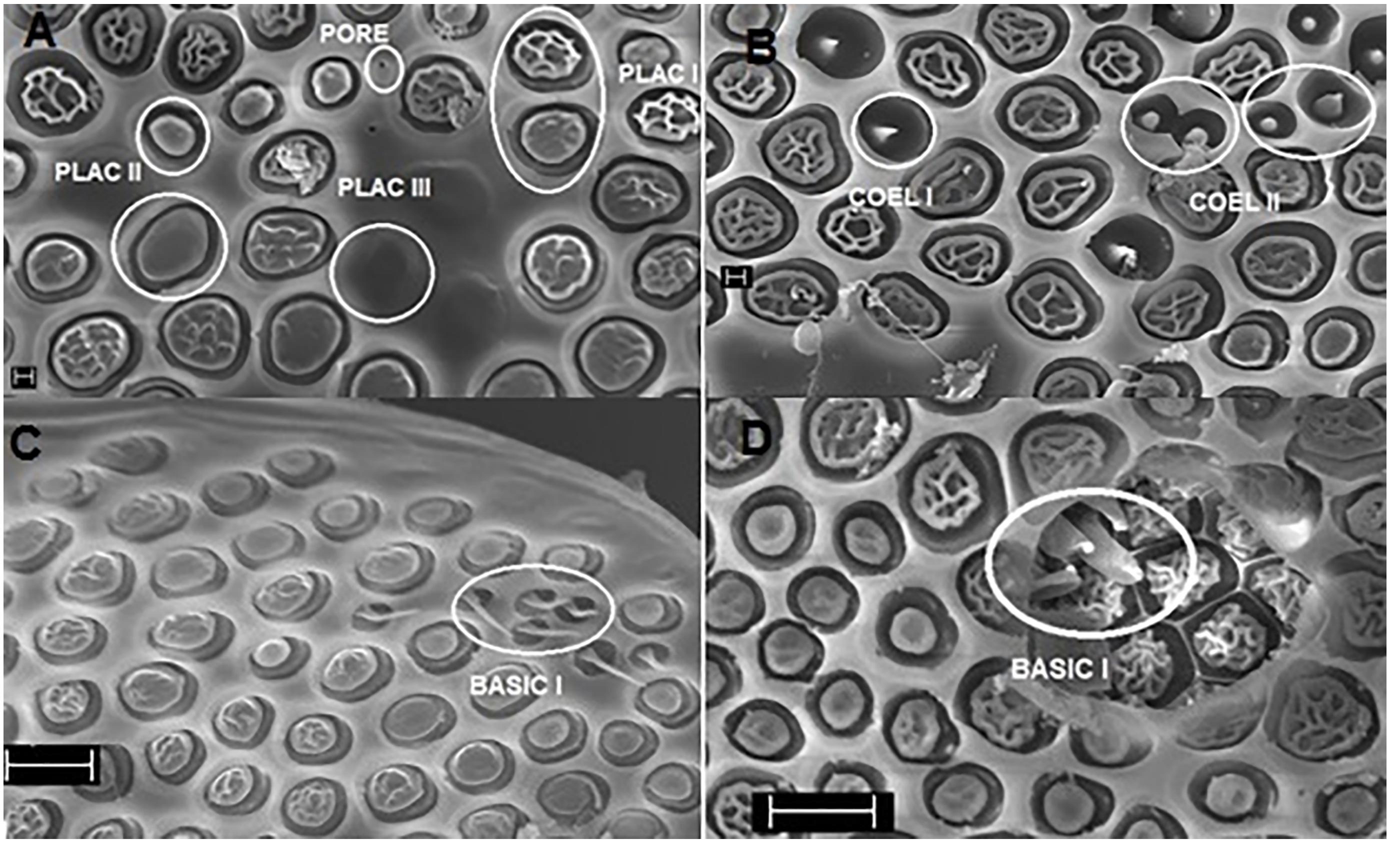

Figure 6. *Cyclocephala putrida*, Burmeister, 1847, antennal sensilla. A) Sensilla placodea type I, II, and III (PLAC I-III), and sensilla ampullacea (PORE). B) Sensilla coeloconica type I and II (COEL I-II). C-D) Sensilla basoconica type I (BASIC I). A-B) Scale 2 micrometers. C-D) Scale: 10 micrometers.

Table 3. Biological data of *Cyclocephala* species.

Cyclocephala species	Duration (days)						reference
	Egg	Fist instar	Second instar	Third instr	Pupae	Egg to adult	
C. melanocephala	17.8	22.3	21.0	38.3	13.3	113.0	Nogueira et al. 2013
C. paraguayensis	18.7	42.2	20.0	75.3	11.4	171.0	Albuquerque et al. 2014
C. tucumana	14.7	47.7	66.1	176.8	15.0	211.2	Nogueira et al. 2013
C. verticalis	17.2	22.0	23.7	150.8	14.8	228.6	Rodrigues et al. 2010
C. celata	13.9	22.0	26.2	68.3	38.0	164.0	Souza et al. 2014

Regarding the immatures, *C. putrida* larvae fed soil organic matter, so the species is not a potential pest to cultivated plants. White grubs had similar behavior compared to other *Cyclocephala* species. Its active third instar is yellowish-white, while prepupa is white and builds a pupal chamber, just as observed in *C. celata* and *C. distincta* (Souza et al. 2014, 2015).

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Author Contribution

Fabiola Gonzaga Saldanha: Contribution to data collection; Contribution to data analysis and interpretation; Contribution to manuscript preparation; Contribution to critical revision, adding intellectual content.

Sérgio Roberto Rodrigues: Contribution to data collection; Contribution to data analysis and interpretation; Contribution to manuscript preparation; Contribution to critical revision, adding intellectual content.

Juares Fuhrmann: Contribution in the analysis and interpretation of data, also assisting in article revision, adding intellectual content.

Ricardo Aparecido Amaro: Contribution to data collection; Contribution to data analysis and interpretation; Contribution to manuscript preparation; Contribution to critical revision.

Conflicts of interest

The authors declare that they have no conflict of interest related to the publication of this manuscript.

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Abstract: The Arctiina, Callimorphina and Spilosomina moths comprise 340 species in the neotropics. Here we provide a list of Arctiina, Callimorphina and Spilosomina species from the Brazilian Amazon. The list was produced from specimens deposited in the most important Brazilian collections and from literature data. We registered 17 species of Arctiina, two of Callimorphina and 16 of Spilosomina. The proportion of Brazilian Amazon Callimorphina (28.6%) and Arctiina (19.1%) in relation to Neotropical fauna were nearly to those found for other Arctiini subtribes in the Brazilian Amazon. However, the Spilosomina records were extremely low, corresponding to only 6.6% of the Neotropical species. Belém, Fonte Boa and Santarém were the municipalities with the highest number of registered species, with 14, 9 and 8, respectively. As this is the last article on the fauna of the Arctiini subtribes of the Brazilian Amazon, we summarize the main patterns observed for the tribe and identify the main knowledge gaps regarding this taxon.

Keywords: *Amazon; Tiger moths; Inventory; Noctuoidea.*

Arctiini Leach, [1815] (Lepidoptera, Erebidae, Arctiinae) da Amazônia Brasileira. V – Subtribos Arctiina Leach, [1815], Callimorphina Walker, [1865] e Spilosomina Seitz, 1910

Resumo: As mariposas Arctiina, Callimorphina e Spilosomina totalizam 340 espécies na região Neotropical. Neste trabalho, nós apresentamos uma lista das espécies de Arctiina, Callimorphina e Spilosomina que ocorrem na Amazônia brasileira. A lista foi produzida através de observação de espécimes depositados nas mais importantes coleções brasileiras e também através de dados da literatura. Foram registradas 17 espécies de Arctiina, duas de Callimorphina e 16 de Spilosomina. A proporção de Callimorphina (28,6%) e Arctiina (19,1%) em relação à fauna neotropical foi semelhante à encontrada para as outras subtribos de Arctiini da Amazônia brasileira. No entanto, os registros de Spilosomina foram extremamente baixos, correspondendo a apenas 6,6% das espécies neotropicais. Belém, Fonte Boa e Santarém foram os municípios com maior número de espécies registradas, com 14, 9 e 8, respectivamente. Como este é o último artigo sobre a fauna das subtribos de Arctiini da Amazônia brasileira, nós resumimos os principais padrões observados para a tribo e identificamos as principais lacunas de conhecimento sobre esse táxon.

Palavras-chave: *Amazônia; Mariposas-tigre; Inventário; Noctuoidea.*

Introduction

There are currently 1,023 Arctiini moths referred to the Brazilian Amazon, including 273 species of Ctenuchina (Teston et al. 2019a), 219 species of Euchromiina (Teston & Ferro 2019), 62 species of Pericopina (Teston & Ferro 2016a) and 469 species of Phaegopterina (Teston & Ferro 2016b). However, it is still not certain how many species of the Arctiina, Calimorphina and Spilosomina subtribes exist in the Brazilian Amazon region.

The Arctiina and Callimorphina moths have a worldwide distribution (Weller et al. 2009). In the neotropic there are seven genera and 89 species of Arctiina and there is one genus and seven species of Callimorphina (Vincent & Laguerre 2014). In adults of Arctiina the size varies from medium to large and the coloring is also varied, with spots and stripes. Male genitalia are simple or reduced. Female pheromone glands can be swollen, divided, and digitated. Larvae have short hair and several species are polyphagous, feeding on plants with pyrrolizidine alkaloids (Weller et al. 2009).

Several Callimorphina species tend to be large and brightly colored, and some are diurnal. The morphology of adults, especially in the male genitalia, varies, and in most of them there is a reduced counter-tympanal hood. Larvae feeding on various herbaceous plants, including plants with pyrrolizidine alkaloids (Weller et al. 2009).

The Spilosomina moths are also cosmopolitan (Schmidt 2009). In the Neotropics there are 23 genera and 244 species (Vincent & Laguerre 2014). Adults are medium to large in size and vary in color. Larvae feed on low-growth herbaceous plants, and are more common in non-forest habitats, typically xeric forests, savannas, prairies, and wetlands (Schmidt 2009).

The caterpillars of some species present in these three sub-tribes are polyphagic and feed on plants of economic importance, but they are not pests of any importance (Schmidt 2009; Weller et al. 2009 and Teston et al. 2019b).

Here, we present a list of Arctiina, Callimorphina and Spilosomina moths occurring in the Brazilian Amazon. In this article we conclude the series of inventories of Arctiini for the Brazilian Amazon (Teston & Ferro 2016ab, Teston & Ferro 2019 and Teston et al. 2019a).

Materials and Methods

We intensively searched the literature and examined specimens from entomological collections of the Instituto Nacional de Pesquisas na Amazônia (INPA; Manaus), Museu Paraense Emilio Goeldi (MPEG; Belém), Coleção Becker (VOB; Camacan), Coleção Entomológica Padre Jesus Santiago Moure of the Universidade Federal do Paraná (DZUP; Curitiba), Fundação Instituto Oswaldo Cruz (FIOC; Rio de Janeiro), Museu de Zoologia of the Universidade de São Paulo (MZUSP; São Paulo), Museu Nacional of the Universidade Federal do Rio de Janeiro (MNRJ; Rio de Janeiro), and Laboratório de Estudos de Lepidópteros Neotropicais (LELN) of the Universidade Federal do Oeste do Pará (UFOPA; Santarém). To identify the species, we used literature (Hampson 1901, 1920, Seitz 1919-1925, Vincent & Laguerre 2014, Watson 1971, 1973) and specimens deposited in the visited collections. The systematic organization to generic level follows Vincent & Laguerre (2014).

The geographical coordinates of the localities in the Brazilian Amazon with Arctiinae records were obtained from the Geo Loc tool of “Species Link date & tools” (http://splink.cria.org.br/geoloc) and Google Earth (https://earth.google.com/web/). The list is organized alphabetically. Species and records without precise location data, and those from locations that belong to more than one biome (e.g., Cerrado and Amazon) were not included in the list.

Results

We registered 17 species of Arctiina, 2 of Callimorphina and 16 of Spilosomina (Table 1). Of the total of 35 species, five were new occurrences for the Brazilian Amazon (three Arctiina and two Spilosomina) (indicated by “NEW” in the Table 1). One Spilosomina species appear as new records for the municipalities and their respective States (indicated by “AMZ”). In total, 41 (5.2%) Amazonian municipalities had moths species records of three Subtribes (Table 2 and Figure 1). Belém (PA), Fonte Boa (AM), and Santarém (PA) were the municipalities with the highest number of species, with 14, 9, and 8, respectively.

We recorded six genera, of which one were monospecific. The genera *Virbia* Walker, 1854 presented the highest number of species (13), followed by *Hypercompe* Hübner, [1819] (8) and *Paracles* Walker, 1855 (7). The species with the highest number of locality records was *Utetheisa ornatrix* (Linnaeus, 1758) (with 14), followed by *Virbia subapicalis* (Walker, 1854) (11), and *Paracles laboulbeni* (Bar, 1873) (10). Eigtheen species (51.4%) occurred in only one locality (Table 1).

Discussion

We consider low the total number of records for these three subtribes for the Brazilian Amazon (35). This value corresponds to only 10.3% of the Neotropical fauna (340, Vincent & Laguerre 2014, Teston et al. 2019b) and are slightly larger than the southest Brazilian State (Rio Grande do Sul, 29, Ferro & Teston 2009), which has an area approximately 18 times smaller and a latitude about 6 times larger than the Brazilian Amazon. Considering the subtribes separately, the proportion of Brazilian Amazon Callimorphina (28.6%) and Arctiina (19.1%) in relation to Neotropical fauna were nearly to those found for other subtribes in the Brazilian Amazon (between 17 and 31%, Teston & Ferro 2016ab, Teston & Ferro 2019, Teston et al. 2019a, Vincent & Laguerre 2014). However, the Spilosomina records were extremely low, corresponding to only 6.6% of the 244 Neotropical species (Vincent & Laguerre 2014). A possible explanation for this low richness would be the difficulty in identifying the Spilosomina species. *Paracles* and *Hypercompe*, for example, are genera with a large number of species with similar color and size patterns. As we considered in our study only the species with no doubtful identification, many species (including *Paracles* and *Hypercompe*) were not included in our list. However, the low number of species in this subtribe in the Amazon may indicate that this taxon is less diverse at low latitudes.

Table 1. Arctiina, Callimorphina and Spilosomina (Erebidae, Arctiinae, Arctiini) species of the Brazilian Amazon. The record column shows the Brazilian state in abbreviated form followed by municipality. The name of the locality is enclosed in braces and the author of the first record is in parentheses. * New record. States abbreviations: AM= Amazonas, AP = Amapá, MA = Maranhão, MT = Mato Grosso, PA = Pará, RO = Rondônia and RR = Roraima.

	Subtribe / Species	Record
	Arctiina Leach, [1815]	
1.	*Pseudalus affinis* Rothschild, 1933	AM, Fonte Boa (Rothschild 1933)
2.	*Pseudalus aurantiacus* Rothschild, 1909	PA, [Belém] (Rothschild 1922)
3.	*Pseudalus leos* (Druce, 1898)	AM, Fonte Boa (Rothschild 1910b); PA, Belém*
4.	*Pseudalus limona* Schaus, 1896	PA, Benevides*, Santarém (Valente et al. 2018)
5.	*Virbia divisa* (Walker, 1854) [NEW]	AM, Fonte Boa*
6.	*Virbia dotata* (Walker, [1865])	AM, Manaus (Hampson 1901), Tefé {Ega} (Walker [1865])
7.	*Virbia egaca* (Walker, [1865])	AM, Tefé {Ega} (Walker [1865])
8.	*Virbia epione* Druce, 1911	PA, Belterra {National Forest of Tapajós} (Freitas 2014)
9.	*Virbia hypophaea* Hampson, 1901	PA, [Belém] (Rothschild 1922)
10.	*Virbia medarda* (Stoll, [1781])	[AM], Amazonas [river] (Hampson 1901); MA, Açailândia*; PA, Belém*, Belterra {National Forest of Tapajós} (Freitas 2014)
11.	*Virbia minuta* (R. Felder, 1874)	[AM], Amazonas river (R. Felder 1874)
12.	*Virbia ovata* Rothschild, 1910 [NEW]	AM, Manaus*; PA, Santarém*
13.	*Virbia palmeri* (Druce, 1911)	PA, [Belém] (Rothschild 1922)
14.	*Virbia parva* Schaus, 1892	PA, [Belém] (Rothschild 1922)
15.	*Virbia satara* Seitz, 1919	AM, Fonte Boa*; RO, Jarú*; PA, Almeirim (Hawes et al. 2009)
16.	*Virbia subapicalis* (Walker, 1854)	AM, Fonte Boa (Rothschild 1910a), Tefé {Ega} (Walker 1854); PA, Altamira (Teston and Delfina 2010), Belterra {National Forest of Tapajós} (Freitas 2014), Itaituba (Rothschild 1910a), Marabá*, Santarém (Valente et al. 2018), São Félix do Xingu {Serra do Pardo National Park} (Teston & Correa 2015); RO, Cacaulândia*, Candeias do Jamari*, Porto Velho*
17.	*Virbia underwoodi* Druce, 1911 [NEW]	PA, Belém*
	Callimorphina Walker, [1865]	
18.	*Utetheisa ornatrix* (Linnaeus, 1758)	AM, Alto Solimões (Travassos 1946), Benjamin Constant (Travassos 1946), São Paulo de Olivença (Travassos 1946), Manaus (Bryk 1953); AP, Santana*; MA, Açailândia*; PA, (Travassos 1946), Altamira (Teston and Delfina 2010), [Belém] (Rothschild 1922), Belterra {National Forest of Tapajós} (Freitas 2014), Chaves (Hampson 1901), Mojuí dos Campos*, Santarém*, São Félix do Xingu {Serra do Pardo National Park} (Teston & Correa 2015), Soure*; RO, Cacaulândia*
19.	*Utetheisa pulchella* (Linnaeus, 1758)	PA, Mojuí dos Campos (Teston et al. 2019b)
	Spilosomina Seitz, 1910	
20.	*Hypercompe abdominalis* (Walker, [1865])	AM, Rio Purus (Bryk 1953), São Paulo de Olivença*; PA, [Belém] (Hampson 1901), Ourém*, Santarém*, Tucuruí*
21.	*Hypercompe alpha* (Oberthur, 1881)	PA, Almeirim (Hawes et al. 2009)
22.	*Hypercompe brasiliensis* (Oberthür, 1881) [AMZ]	AM, [Santa Isabel do Rio Negro] {Rio Preto}*
23.	*Hypercompe cunigunda* (Stoll, [1781])	AM, São Gabriel da Cachoeira {Querari}*, São Paulo de Olivença*; MA, Açailândia*; PA, [Belém] (Rothschild 1922), Marabá*, São Félix do Xingu {Serra do Pardo National Park} (Teston & Correa 2015); RO, Cacaulândia*; RR, Alto Alegre*
24.	*Hypercompe detecta* (Oberthür, 1881)	PA, [Belém] (Oberthür 1881)
25.	*Hypercompe laeta* (Walker, 1855) [NEW]	MT, Sinop*
26.	*Hypercompe marcescens* (R. Felder, 1874)	[AM], Amazonas river (R. Felder 1874)
27.	*Hypercompe turruptianoides* (Rothschild, 1910)	AM, [Atalaia do Norte] {Santo Antônio do Javari} (Rothschild 1910a)
28.	*Isia alcumena* (Berg, 1882)	PA, Altamira (Delfina and Teston 2013); RO, Cacaulândia*
29.	*Paracles brunnea* (Hübner, [1831]) [NEW]	PA, Santarém*
30.	*Paracles contraria* Walker, 1855	AP, Serra do Navio*; PA, [Belém] (Walker 1855), Capitão Poço*, Santarém (Hampson 1901), Viseu*
31.	*Paracles juruana* (Butler, 1878)	AM, [Atalaia do Norte] {Santo Antônio do Javari} (Rothschild 1910b), [Carauari] Rio Juruá (Butler 1878), Codajás (Rothschild 1910b), Fonte Boa (Rothschild 1910b), Manacapuru to Tefé (Rothschild 1910b); RO, Porto Velho {Calama} (Rothschild 1910b)
32.	*Paracles klagesi* (Rothschild, 1910)	AM, Fonte Boa (Rothschild 1910b)
33.	*Paracles laboulbeni* (Bar, 1873)	AM, [Iranduba] {Lago Janauarí, Rio Negro}(Adis 1983), [Manaquiri] {Lago Janauacá, Rio Solimões} (Adis 1983), [Manaus] {Manaus-Itacoatiara highway AM-010, Km 21 and Km 45}(Adis 1983); PA, Altamira (Teston and Delfina 2010), [Belém] (Rothschild 1922), Gur[u]pá (Hampson 1901), [Prainha] (Butler 1878), Santarém*, São Félix do Xingu {Serra do Pardo National Park} (Teston & Correa 2015)
34.	*Paracles sericea* (Schaus, 1896)	AM, Itacoatiara (Bryk 1953), Parintins (Bryk 1953)
35.	*Paracles tenuis* (Berg, 1877)	AM, [Atalaia do Norte] {Santo Antônio do Javari} (Rothschild 1910b), Codajás (Rothschild 1910b), Fonte Boa (Rothschild 1910b), Tefé to Fonte Boa (Rothschild 1910b); PA, [Belém] (Rothschild 1922); RO, Porto Velho {Calama} (Rothschild 1910b)

[AMZ] Species recorded for states within the Amazon biome by Ferro and Diniz (2010), but without precise location and biome information, and Amazon biome by Ferro and Diniz (2007). So these species are new records for the municipalities. [NEW] New record for the Brazilian Amazon.

Table 2. Geographic coordinates of municipalities and richness of the Arctiina, Callimorphina and Spilosomina species (Erebidae, Arctiinae, Arctini) in the Brazillian Amazon Biome.

N°	State	Municipality	Richness	Latitude	Longitude
1	AP	Santana	1	00°03'30"S	51°10'54"W
2	AP	Serra do Navio	1	00°53'45"N	52°00'07"W
3	AM	Atalaia do Norte	3	04°25'46"S	70°15'41"W
4	AM	Benjamin Constant	1	04°22'60"S	70°01'52"W
5	AM	Carauari	1	05°03'01"S	66°56'59"W
6	AM	Codajás	2	03°50'13"S	62°03'25"W
7	AM	Fonte Boa	9	02°30'51"S	66°05'30"W
8	AM	Iranduba {Lago Janauarí}	1	03°12'45"S	60°01'52"W
9	AM	Itacoatiara	1	03°08'36"S	58°26'39"W
10	AM	Manaquiri {Lago Janauacá}	1	03°23'22"S	60°18'31"W
11	AM	Manaus	5	03°06'07"S	60°01'30"W
12	AM	Parintins	1	02°37'42"S	56°44'08"W
13	AM	Santa Isabel do Rio Negro	1	00°24'51"S	65°01'08"W
14	AM	São Gabriel da Cachoeira {Querari}	1	01º04'60"N	69°50'59"W
15	AM	São Paulo de Olivença	3	03°22'42"S	68°52'20"W
16	AM	Tefé	3	03°21'16"S	64°42'40"W
17	MA	Açailândia	3	04°56'49"S	47°30'17"W
18	MT	Sinop	1	11°52'51"S	55°30'08"W
19	PA	Almeirim	2	01º31'24"S	52º34'54"W
20	PA	Altamira †	4	03º11'55"S	52º10'15"W
21	PA	Belém	14	01°27'21"S	48°30'15"W
22	PA	Belterra {National Forest of Tapajós}†	4	03°01'05"S	54°58'10"W
23	PA	Benevides	1	01°21'42"S	48°14'40"W
24	PA	Capitão Poço	1	01°44'48"S	47°03'33"W
25	PA	Chaves	1	00°09'36"S	49°59'18"W
26	PA	Gurupá	1	01°24'18"S	51°38'23"W
27	PA	Itaituba	1	04°16'35"S	55°59'01"W
28	PA	Marabá	2	05°22'07"S	49°07'04"W
29	PA	Mojuí dos Campos†	2	02°41'44"S	54°34'14"W
30	PA	Ourém	1	01°33'07"S	47°06'52"W
31	PA	Prainha	1	01°47'60"S	53°28'47"W
32	PA	Santarém	8	02°26'36"S	54°42'29"W
33	PA	São Félix do Xingu {Serra do Pardo National Park}†	4	05º46'26"S	52°37'13"W
34	PA	Soure	1	00°43'01"S	48°31'24"W
35	PA	Tucuruí	1	03°42'01"S	49°42'00"W
36	PA	Viseu	1	01°11'49"S	46°08'23"W
37	RO	Cacaulândia	4	10°20'21"S	62°53'43"W
38	RO	Candeias do Jamari	1	08°48'35"S	63°41'44"W
39	RO	Jarú	1	10°26'20"S	62°27'58"W
40	RO	Porto Velho	3	08°45'43"S	63°54'13"W
41	RR	Alto Alegre	1	02°53'45"N	61°29'51"W

Geographic coordinates of municipality marked with † are the citations referred, other obtained by Google Earth or Geo Loc tool (see Materials and Methods).

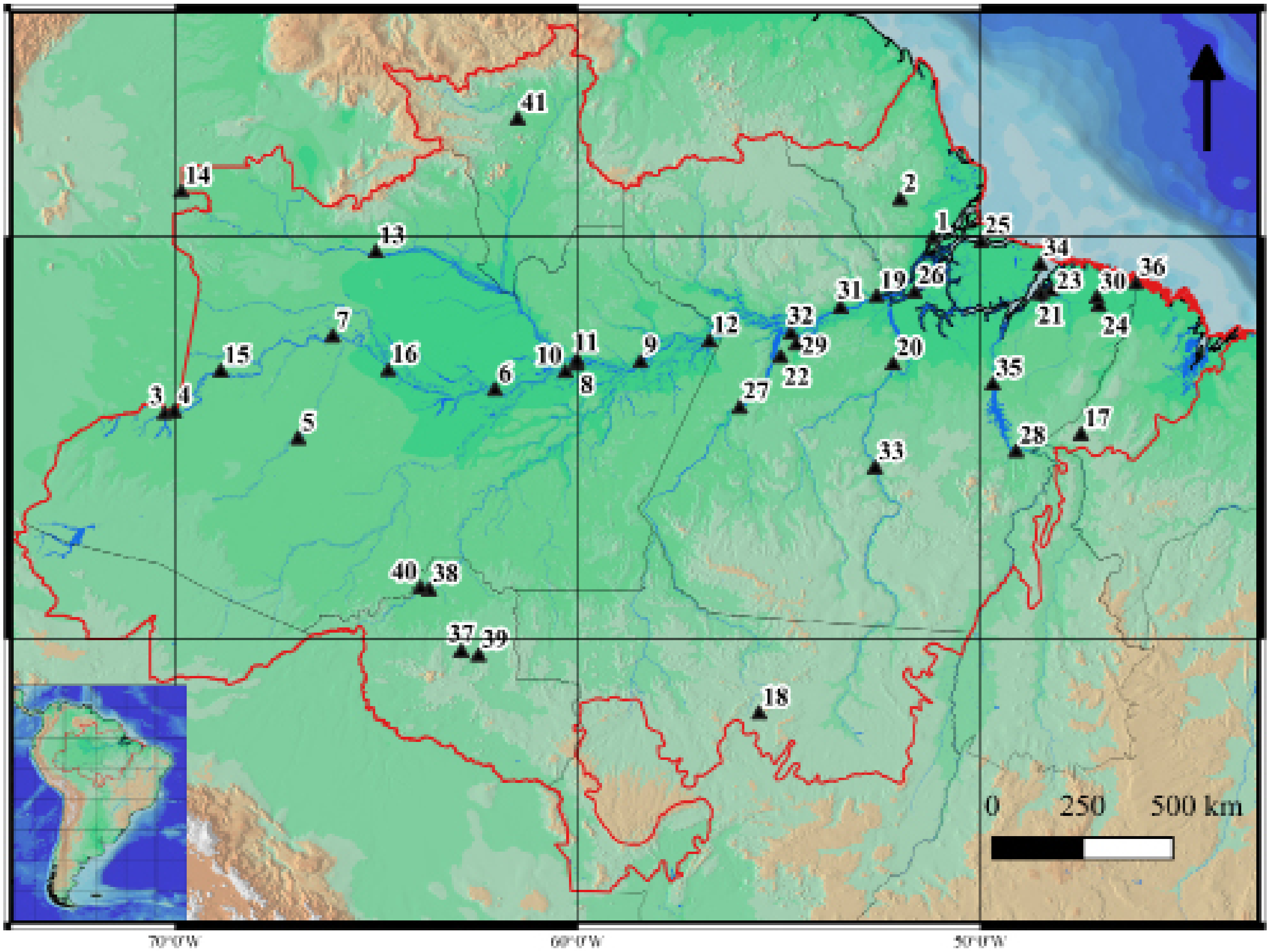

Figure 1. Geographic distribution of the Arctiina, Callimorphina and Spilosomina species records in the Brazillian Amazon. The numbers refer to the municipalities of Table 2.

In a Peruvian site (latitude 13S), for example, only 5 species of Spilosomina were sampled (Grados, 2002), but in the southernmost state of Brazil (Rio Grande do Sul, latitude about 30S) were recorded 26 species of Spilosomina (Ferro & Teston 2009), 10 more species than the entire Amazon biome. Nevertheless, other samplings carried out at higher latitudes (between 22 and 32S) (but with a much smaller sample effort) found a low number of Spilosomina species: 7 in the Bosque Serrano de la provincia de Córdoba, Argentina (Beccacece et al. 2012), and 5 in the Chaco Serrano and Yungas Ecoregions, also in Argentina (Beccacece et al. 2016).

The highest richness for the three subtribes (Arctiina, Callimorphina and Spilosomina together) in an Amazonian site were 14, 9 and 8 species (Belém, Fonte Boa and Santarém, respectively). These values are similar to those found in well-sampled Atlantic Forest sites, as Boracéia, (10 species, Ferro & Diniz 2007) and Joinville (11, Ferro et al. 2012). In the Cerrado well-sampled sites, however, were recorded only seven (Scherrer et al. 2013) and six (Moreno & Ferro 2016) species of these three subribes.

With this paper we concluded the series of faunal inventories about Brazilian Amazon Arctiini (Teston & Ferro 2016a, b, Teston & Ferro 2019, Teston et al. 2019a). Some common patterns could be observed in these papers. First, there was a great overlap in relation to the locations with the highest number of Arctiini species records. Belém, the capital of the state of Pará, was among the three most diverse locations in all Arctiini inventory papers (Teston & Ferro 2016a, b, Teston & Ferro 2019, Teston et al. 2019a, this work), followed by Santarém (Teston & Ferro 2019, Teston et al. 2019a, this work), Cacaulândia (Teston & Ferro 2016a, Teston et al. 2019a) and Fonte Boa (Teston & Ferro 2016a, this work). This pattern was probably related to historical sampled effort (several naturalists sampled in the Amazon in the 19th and 20th centuries), and to the proximity/easer access to sampled sites and research institutions. Second, the number of Brazilian Amazon arctiid records was underestimaded for all subtribes. The tiger moth fauna was never sampled in the vast majority of the biome.

The Legal Amazon comprises 775 muncipalities and there are sampled records in only 41 for Arctiina, Callimorphina and Spilosomina (this work), 48 for Pericopina (Teston & Ferro 2016b), 69 for Phaegopterina (Teston & Ferro 2016a), 71 for Euchromiina (Teston & Ferro 2019), and 75 for Ctenuchina (Teston et al. 2019a). Besides, the sampling points are poorly distributed and usually restricted to more populated cities and along major rivers. In addition, the majority of the locations were insufficiently sampled, as observed by the large proportion of sites with only one species record (e.g. 58.5% for Arctiina, Callimorphina and Spilosomina). Third, the Arctiini fauna was more diverse in humid than in xeric biomes. There are currently 1,058 Arctiini moths registred to the Brazilian Amazon, considering the subtribes Ctenuchina, Euchromiina, Pericopina and Phaegopterina (Teston & Ferro 2016a, b, 2019, Teston et al. 2019a) and those listed in this study. The Arctiini richness of the the Brazilian Amazon is high but slightly lower than the Atlantic Forest (1,193 species, Ferro & Melo 2011). This suggest a pattern of higher diversity in humid forest environments since in the Cerrado biome there are only 595 Arctiini recorded species (Ferro & Diniz 2010).

In order to suggest efficient conservation policies for the Amazon Arctiini fauna, it is urgent to intensify the sampling effort in this biome, both spatially (more locations, several strata and types of vegetation), and temporally (sampled also during the day, during several months/years, throughout all nocturnal period). Besides, it is necessary a higher investment in taxonomy/systematic research on the Neotropical Arctiinae fauna to reduce the Linnean shortfalls. Finally, we think it is important to expand the studies for the Lithosiini tribe and to understand in more depth the issue of the low richness of Spilosomina in the Amazon.

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Author Contributions

José A. Teston and Viviane G. Ferro: Substantial contribution in the concept and design of the study.

Conflicts of Interest

The authors declare that they have no conflict of interest related to the publication of this manuscript.

Data availability

The data are deposited in the respective collections mentioned in the Material and Methods.

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Short Communication

First record of plastic ingestion by an important commercial native fish (*Prochilodus lineatus*) in the middle Tietê River basin, Southeast Brazil

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Abstract: This is the first study to report the plastic ingestion by *Prochilodus lineatus* in Brazilian fluvial ecosystems. We examined 32 individuals collected in two contrasting lotic environments: the highly polluted Tietê River and its much less degraded tributary, Peixe River. Most individuals, 71.88%, contained plastic in their digestive tract, with averages of 3.26 and 9.37 particles per individual in the tributary and main river, respectively. The blue color was predominant among the observed plastic particles and size ranged from 0.18 to 12.35 mm. Plastic ingestion must be accidental, predominantly, since the species has an iliophagous eating habit. As this species is regionally the most important fishery resource, potential adverse effects of this type of contamination may be transferred to human consuming populations. Mitigation measures against pollution are urgent in the Tietê River basin.
Keywords: Curimbatá; Digestive tract content; Plastic contamination; River pollution.

Primeiro registro de ingestão de plástico por um importante peixe comercial nativo (*Prochilodus lineatus*) na bacia do médio rio Tietê, sudeste do Brasil

Resumo: Este é o primeiro estudo a relatar a ingestão de plástico por *Prochilodus lineatus* em ecossistemas fluviais brasileiros. Nós examinamos 32 indivíduos coletados em dois ambientes lóticos contrastantes: o rio Tietê, altamente poluído, e seu tributário muito menos degradado, o rio do Peixe. A maioria dos indivíduos, 71,88%, continha plástico em seus tratos digestivos, com médias de 3,26 e 9,37 partículas por indivíduo no tributário e no rio principal, respectivamente. A cor azul foi predominante entre as partículas plásticas observadas e o tamanho variou de 0,18 a 12,35 mm. A ingestão de plástico deve ser predominantemente acidental, uma vez que a espécie possui um hábito alimentar iliófago. Como esta espécie constitui o recurso pesqueiro mais importante regionalmente, potenciais efeitos adversos desse tipo de contaminação podem ser transferidos para populações humanas consumidoras. Medidas de mitigação contra a poluição são urgentes na bacia do rio Tietê.
Palavras-chave: *Contaminação plástica; Conteúdo do trato digestivo; Curimbatá; Poluição dos rios.*

Introduction

Plastics are synthetic polymers that provide uncountable benefits to modern society. However, its massive consumption associated with improper disposal in nature has been the source of a serious and widespread contamination (Silva-Cavalcanti et al. 2017, Blettler et al. 2018, Olivatto et al. 2018).

The accumulation of plastic particles, especially in the aquatic ecosystems, has increased considerably over the last decades. Recent estimates suggest that about 4.9 billion tons of this material is found in natural environments (Geyer et al. 2017). In the oceans alone, millions of tons of plastic are dumped every year, mostly, approximately 80%, transported by rivers (Jambeck et al. 2015).

In the rivers, once fragmented into smaller particles, these polymers can be ingested, intentionally or accidentally, by many animals, especially the ichthyofauna (Lusher et al. 2013, 2015, Cardozo et al. 2018, Azevedo-Santos et al. 2019). In Brazil, the presence of plastic in the digestive tract of fish was first recorded in estuarine ecosystems (Possatto et al. 2011, Dantas et al. 2012, Ramos et al. 2012) and then in freshwater ones (Silva-Cavalcanti et al. 2017, Andrade et al. 2019).

The fish species *Prochilodus lineatus* (Valenciennes 1837), popularly known as "curimba" or "curimbatá", is widely distributed in freshwater environments of the Neotropical region (Castro & Vari 2003, Avigliano et al. 2017). In general, it is an important fishery resource in Southeast Brazil (Novaes & Carvalho 2009, Maruyama et al. 2010, Novaes & Carvalho 2013), but catches have declined in the dammed (hydropower reservoirs) stretches of large rivers (David et al. 2016). The first report of plastic ingestion for this species was provided by Pazos et al. (2017), based on analyzes of individuals from La Plata River (Argentina), and more recently by Blettler et al. (2019), for the middle Paraná River, also in Argentina.

In this paper, we report the first case of plastic ingestion by *P. lineatus* in Brazilian rivers, from individuals of lotic (undammed) stretches of the middle Tietê River basin, where the species is regionally the main fishery resource. The aim of this study was to analyze and compare fish plastic ingestion from contrasting environments in terms of water pollution. Our hypothesis was to find a higher microplastic ingestion in individuals from the Tietê River, given by the strong environmental degradation of this river (which flows through the largest megalopolis of the Southern Hemisphere).

Material and Methods

The study area is in the middle Tietê River basin. The region is located between the municipalities of Laranjal Paulista and Anhembi, São Paulo State, Southeastern Brazil, which includes the Tietê River and important tributaries such as the Peixe River and the Sorocaba River, on the left bank, and the Capivari River, on the right bank (Figure 1).

Thirty-two specimens of *Prochilodus lineatus*, 13 from the Tietê River and 19 from the Peixe River, were analyzed. Fish were collected in April 2019 (IBAMA/SISBIO permanent sampling license to MGN: 13794-1) using gill nets disposed for about 15 hours (overnight). Complementary, some individuals were also obtained from local professional fishermen. After being euthanized with an overdose (more than 283.55 mgL^{-1}) of anesthetic (eugenol) (Vidal et al. 2008), the fish were fixed in 10% formalin and stored in 70% ethanol. All organisms used in the study are deposited in the ichthyological collection of the Laboratório de Biologia e Genética de Peixes (LBP), Universidade Estadual Paulista (UNESP), *campus* of Botucatu, São Paulo, Brazil (lots: LBP 29184; LBP 29185).

In the laboratory, individual digestive tracts were removed through a longitudinal incision in the abdominal region with the use of a scalpel. The entire digestive content of each animal was rinsed with 70% absolute ethylic ethanol (L. S. Chemicals and Pharmaceuticals) in a 50 μm mesh sieve and then transferred to Petri dishes. To avoid contamination during sample processing, we used cotton lab coats and chirurgical gloves, all work surfaces and utensils were alcohol sterilized, and Petri dishes were covered with glass during visual identification (protocols according to Silva-Cavalcanti et al. 2017). Additionally, a Petri dish filled with ethanol was left open over the laboratory bench, adjacent to the microscopy, and examined in the end of every analysis session, for quantification of eventual airborne contamination.

Under a stereomicroscope, plastic particles were separated from organic particles, counted, measured and classified by color. Identification followed visual criteria to determine if the particles were synthetic: absence of cellular or organic structures, clear and homogeneous colors, and fibers evenly thick throughout their entire length (Norén 2007, Hidalgo-Ruz et al. 2012). To confirm the plastic nature of the particles, we performed a peroxide hydrogen (also adding Fe II as catalyzer) digestion test, which is indicated to oxidize organic matter (Prata et al. 2019).

The largest longitudinal dimension was considered to determine the size of the plastic particles. Plastic intake was characterized per sampling location, number of individuals who ingested plastic and number of particles in the digestive tract of each animal.

Results

Most analyzed fish (71.88%) ingested plastic, corresponding to 15 individuals from Peixe River and eight individuals from Tietê River. Forty-nine particles were found in specimens collected in the tributary and 75 in those from the main river, with respective mean values (and standard deviations) per individual of 3.26 (± 1.75) and 9.37 (± 8.19) (Figure 2).

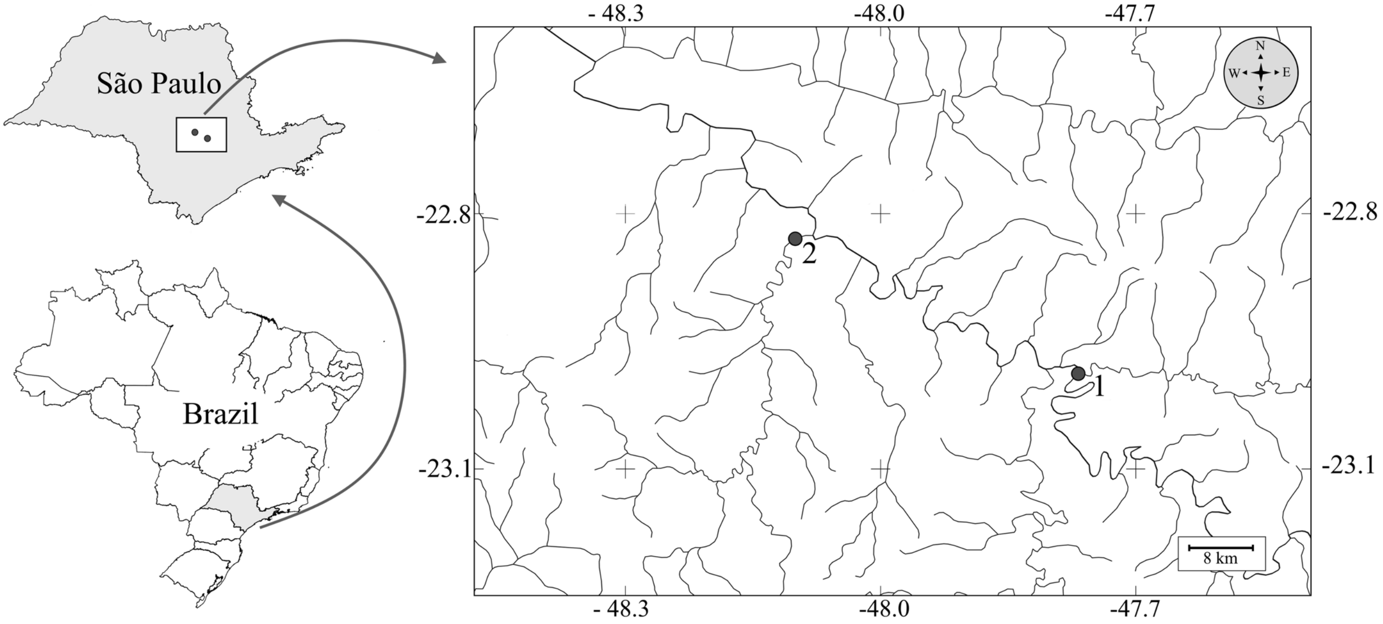

Figure 1. Study area location – middle Tietê River basin, Southeast Brazil. Black circles indicate the sampling sites: (1) Tietê River; and (2) Peixe River.

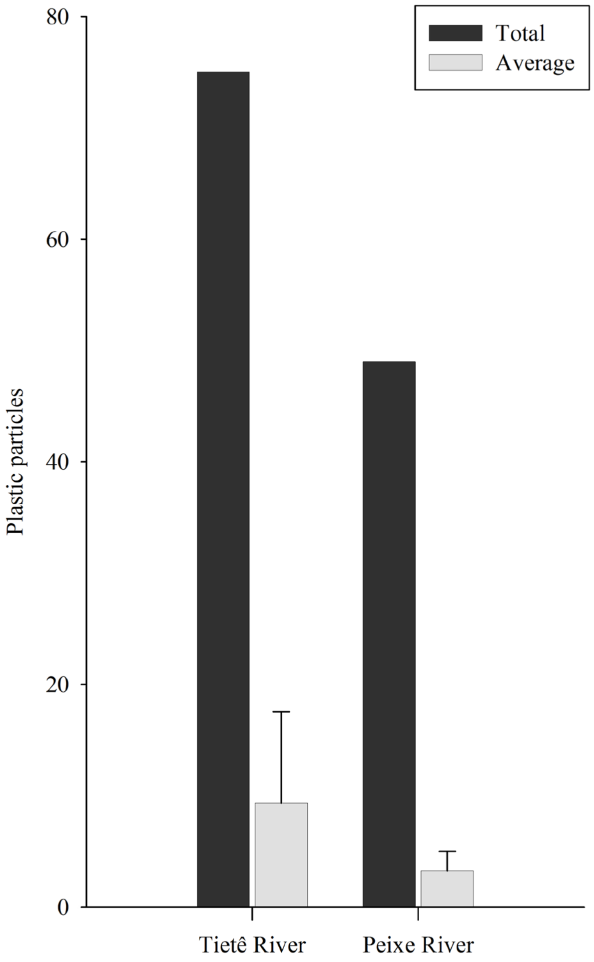

Figure 2. Total and average per individual (± SD) of plastic particles found in *Prochilodus lineatus* from middle Tietê River basin.

Particles presented eight distinct colors (yellow, blue, white, black, pink, transparent, green and red), with predominance of blue in both rivers (61.2% in Peixe River and 68% in Tietê River) (Figure 3, 4), and their sizes ranged from 0.18 mm to 12.35 mm, with 44.44% larger than 1 mm (Figure 3).

Discussion

This work is the first to record plastic particles ingestion by *Prochilodus lineatus* in Brazil. Studies on plastic ingestion by freshwater fish are still scarce in Central and South America, including the Brazilian territory.

Most individuals we analyzed (71.88%) contained micro and mesoplastic in their digestive tracts. Studies show that the percentage of contaminated individuals is highly variable, depending on the considered ecosystem. In Brazil, moderate rates of plastic ingestion, 7.9% to 23%, were reported for estuarine environments (Possatto et al. 2011, Dantas et al. 2012, Ramos et al. 2012), in contrast with the remarkable value of 83%, for fish from an urban river (Silva-Cavalcanti et al. 2017). Andrade et al. (2019) observed a rate of ingestion of 25% for Xingu River, what we think is high, considering that this is an Amazonian river, a region scarcely occupied by human populations. Two other studies that also analyzed plastic ingestion by *P. lineatus*, both in highly polluted rivers in Argentina, observed that 100% of the analyzed individuals had ingested plastic particles (Pazos et al. 2017; Blettler et al. 2019).

It is noticeable in our results the fact that the degree of individual contamination (mean of plastic particles) was about three times higher in the Tietê River compared to its tributary, Peixe River. The number of particles per individual in Tietê River, 9.37, is very high, even when compared to the amount found by Silva-Cavalcanti et al. (2017), 3.6 particles per individual, for fish captured in a river crossing a city in northeast Brazil.

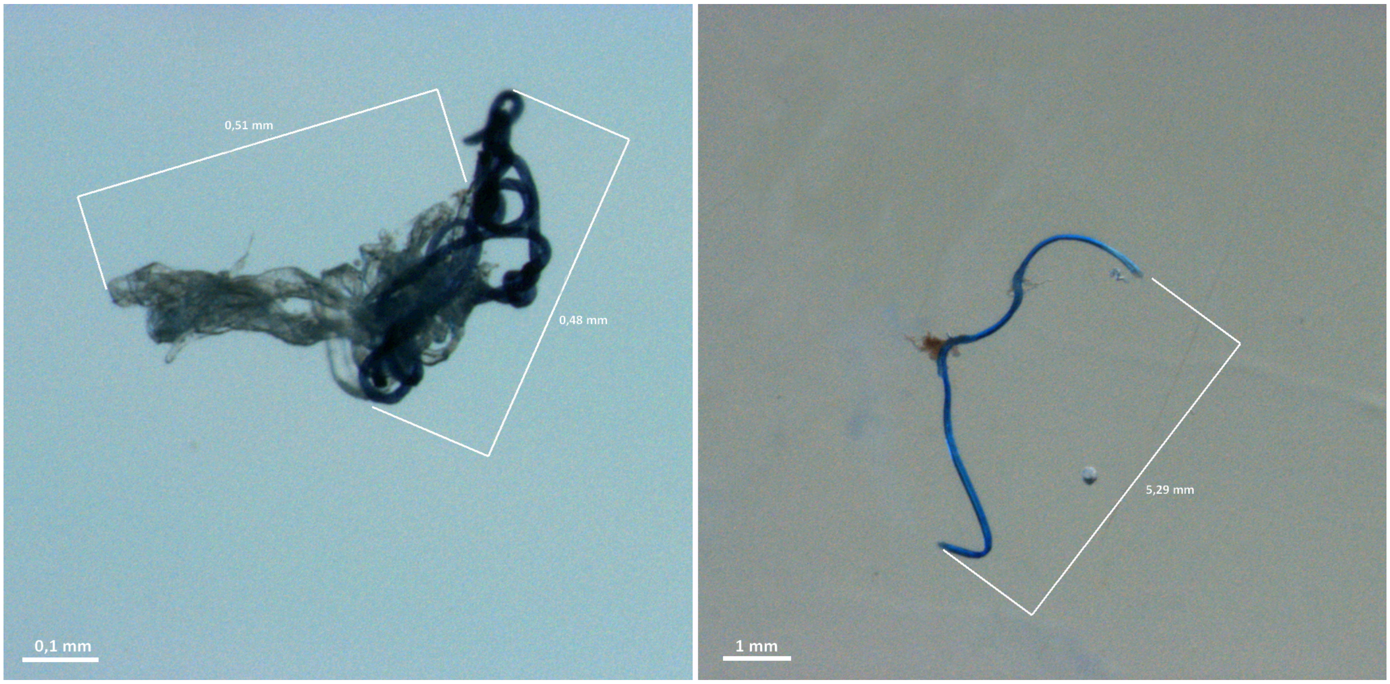

Figure 3. Examples of plastic particles, originated from irregularly dumped solid wastes, found in the stomachs of *Prochilodus lineatus* individuals from middle Tietê River basin.

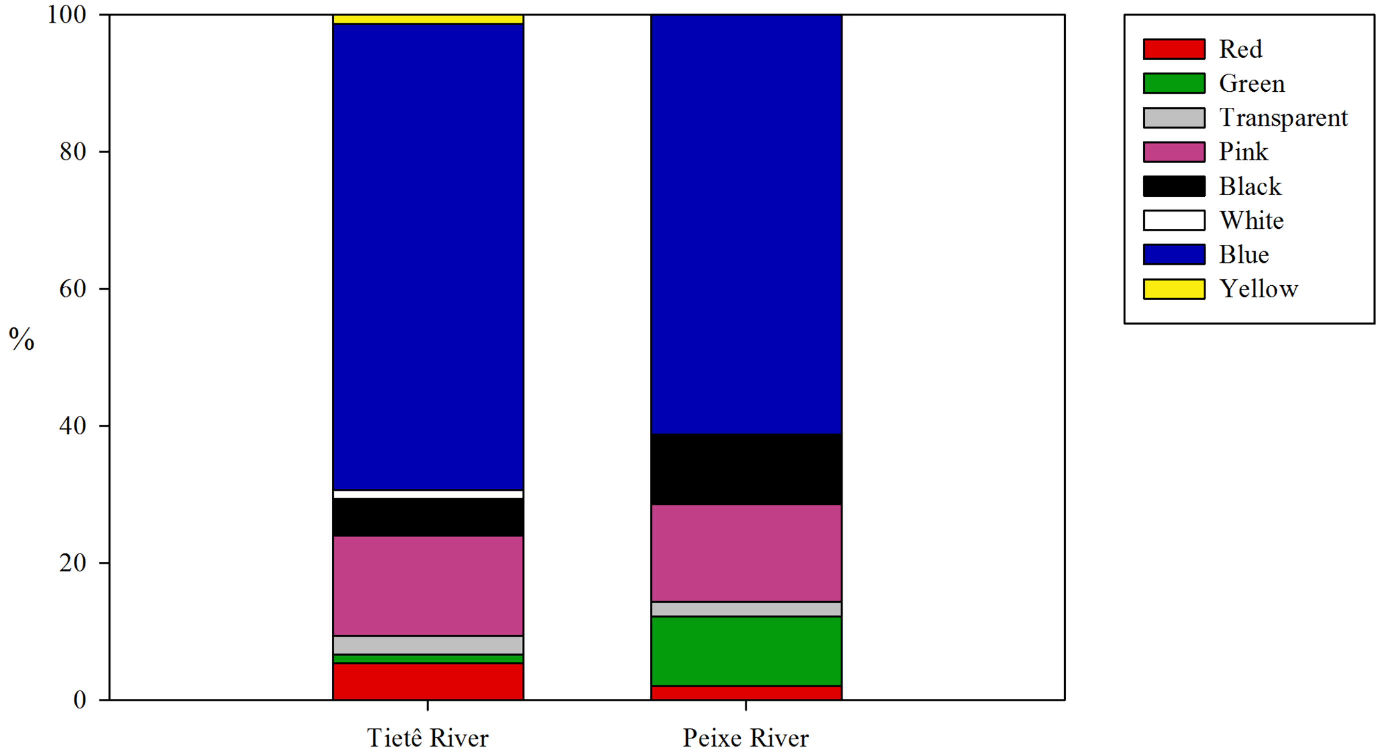

Figure 4. Percentage variation of the different plastic particles in relation to the observed color pattern, found in the stomachs of *Prochilodus lineatus* individuals from middle Tietê River basin.

However, the mean value of ingested of plastic particles by *P. lineatus* from the middle Paraná River in Argentina, 9.9 (Blettler et al. 2019), was even higher than in Tietê River.

Results corroborate our initial hypothesis, explained by the fact that the stretch of the Tietê River upstream the studied area is directly affected by the huge metropolis of São Paulo, with 19.6 million inhabitants (IBGE 2010) (last official census). The river receives, for more than a century, an enormous amount of solid waste, as well as domestic and industrial effluents (Tundisi et al. 2008, Tundisi 2018, Buckeridge & Ribeiro 2018). Other tributaries of the Tietê River, such as Pinheiros River, in the São Paulo city, are also heavily polluted and certainly contribute to the input of synthetic polymers into the main course.

The plastic particles size we observed ranged from 0.18 to 12.35 mm, which includes from micro to mesoplastic. Our minimum values are below the amplitude commonly found for other marine and freshwater ecosystems (Liboiron et al. 2019; Andrade et al. 2019), which could have influenced in the high rate of contamination we report. According to Song et al. (2015), visual identification of microplastics using a microscope is a reliable method for identifying particles > 1 mm. What could explain the small size of the plastic particles we found is the fact that this material may have been ingested from the sediments (see below). Plastic deposited for a long time in the bottom of rivers is probably highly fragmented.

The target species, *P. lineatus*, is well known to move dozens of kilometers, especially for reproductive migration (Agostinho et al. 2003, Capeleti & Petrere Jr. 2006, Stassen et al. 2010). The fact that the analyzed individuals from Peixe River were sampled only a few kilometers upstream the mouth, make us to consider the possibility that the plastic ingestion might have occurred in Tietê River, just before captures.

Another important characteristic of *P. lineatus* is the eating habit of the iliophagous type (Moraes et al. 1997), which consists in the behavior of searching for benthic invertebrates and organic detritus as food items in the rivers bottom. In this work, we did not evaluate the presence of plastic in the sediment, but much probably this was the source of contamination and certainly should be considered in future studies. Blettler et al. (2019) found an extremely high deposition of microplastic particles, reaching 12.687 per m^2, in shoreline sediment samples from polluted Argentine river environments.

As many studies around the world we also verified the predominance of blue color among ingested plastics (*e.g.* Possatto et al. 2011, Ramos et al. 2012, Dantas et al. 2012, Alomar et al. 2017, Ory et al. 2017, Pazos et al. 2017, Bessa et al. 2018, Chagnon et al. 2018, Compa et al. 2018, Digka et al. 2018, Blettler et al. 2019). The different fish species may have a predilection for that color or most plastic pollution is composed by blue material. This second possibility seems to be more consistent to explain our results, once *P. lineatus* feeds on the bottom sediments were discrimination of the food items by color is difficult due to the limitation of light penetration. Again, the characterization of plastic particles deposition in the water column and sediments is a necessary investigation for a better understanding of this environmental problem.

The species *P. lineatus* constitutes an important resource for the regional professional fishery (Novaes & Carvalho 2009, Maruyama et al. 2010, Novaes & Carvalho 2013, David et al. 2016), which are commercialized both locally and in the metropolitan region of São Paulo. In addition to the effects of plastic contamination on fish, the health authorities should be concerned about potential adverse effects that may affect human consumers. Plastic particles can be vectors of microorganisms and also adsorb various chemical contaminants

(Paul-Pont et al. 2018), such as phthalates and bisphenol, endocrine disruptors commonly used in the manufacture of this kind of material, as well as heavy metals and persistent organic pollutants (POPs) (Rios et al. 2007, Massos & Turner 2017, Olivatto et al. 2018). However, the degree of biomagnification of plastics particles in the aquatic food chains and the adverse effects on other consumers such as birds and humans is currently poorly understood (Pegado et al. 2018).

The contamination we have demonstrated shows that additional studies should continue, such as the application of spectrometry techniques to discriminate the kinds of polymers. Other components of the regional biota (*e.g.* plankton and benthos) must be evaluated too, for a complete understanding of the problem. Control measures against solid waste pollution in the Tietê River basin are urgent. In fact, the government of São Paulo State has recently started an environmental recovery program for Pinheiros River, a tributary within the watershed (Folha 2019). That is an excellent initiative, but actions should be extended to the entire river basin.

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Author Contributions

Bruna Q. Urbanski: Substantial contribution in the concept and design of the study; Contribution to data collection; Contribution to data analysis and interpretation; Contribution to manuscript preparation; Contribution to critical revision, adding intellectual content.

Ana C. Denadai: Contribution to data collection; Contribution to data analysis and interpretation; Contribution to manuscript preparation; Contribution to critical revision, adding intellectual content.

Valter M. Azevedo-Santos: Contribution to manuscript preparation; Contribution to critical revision, adding intellectual content.

Marcos G. Nogueira: Substantial contribution in the concept and design of the study; Contribution to data collection; Contribution to manuscript preparation; Contribution to critical revision, adding intellectual content.

Conflicts of Interest

The authors declare that they have no conflict of interest related to the publication of this manuscript.

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Short Communication

A solitary rough-toothed dolphin (Steno bredanensis) in a superport area on northern Rio de Janeiro State, SE Brazil

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Abstract: The solitary behavior is an unusual and peculiar behavior reported in a few sociable dolphin species. This study presents the solitary behavior of an adult rough-toothed dolphin, *Steno bredanensis*, in the Açu Superport (21º49'S; 041º00'W), northern Rio de Janeiro State, SE Brazil. Data about the dolphin were obtained from mobile phone videos and reports by local professionals. Probably, the same dolphin was there since 2017-2018 up to April 2020 (at least). In general, the solitary dolphin swims next to supply boats navigating along the port internal channel, and it rarely continues towards open sea. The dolphin plays with buoys and containment barriers, rubs itself against hulls, and bow-rides and jumps. There is no report of people swimming with this solitary dolphin, touching it, or offering it food in the Açu Superport. Thus, there seems to be no threats to the animal's integrity locally.

Keywords: *Solitary Dolphin; Behavior; Delphinidae; Atlantic Ocean.*

Um golfinho de dentes-rugosos (*Steno bredanensis*) solitário em área de superporto no norte do Estado do Rio de Janeiro, SE Brasil

Resumo: O comportamento solitário é um comportamento incomum e peculiar relatado em poucas espécies sociáveis de golfinhos. Este estudo apresenta o comportamento solitário de um golfinho de dentes-rugosos adulto, *Steno bredanensis*, no Superporto do Açu (21º49'S; 041º00'W), norte do estado do Rio de Janeiro, SE Brasil. Os dados sobre o golfinho foram obtidos a partir de vídeos de telefones móveis e relatos de profissionais locais. Provavelmente, o mesmo golfinho estava lá desde 2017-2018 até abril de 2020 (pelo menos). Em geral, o golfinho solitário nada próximo aos barcos de apoio enquanto eles navegam pelo canal interno do porto, e raramente continua em direção ao mar aberto. O golfinho brinca com boias e barreiras de contenção, esfrega-se contra os cascos e executa comportamentos de saltos. Não há relato de pessoas nadando com esse golfinho solitário, tocando nele ou oferecendo comida a ele no Superporto do Açu. Dessa forma, parece que não há ameaças à integridade do animal localmente.

Palavras-chave: *Golfinho Solitário; Comportamento; Delphinidae; Oceano Atlântico.*

Introduction

Solitary dolphins are social species living apart from their own groups, with little or even no contact with conspecifics, and a high tendency to socialize with humans in different levels, such as touching, social, sexual and playing behaviors (Wilke et al. 2005). Wilke et al. (2005) raised some reasons to explain why social dolphins become solitary and sociable towards people (e.g., food availability, loss of habitat, lack of connectivity between dolphin populations, predation risk, and dolphin's personality); but until now these reasons remain somewhat speculative. Nunny & Simmonds (2019) summarized the current knowledge regarding solitary delphinids and monodontids (ten different species), detailing cases recorded since 2008 worldwide. The most frequent species are the bottlenose dolphins, *Tursiops truncatus* (Montagu 1821) and *T. aduncus* (Ehrenber 1833). More recently, the solitary behavior of the rough-toothed dolphin, *Steno bredanensis* (Lesson 1828), was reported from a juvenile male near a Brazilian Navy base in Sepetiba Bay (22º54'S-23º04'S), southern Rio de Janeiro State, SE Brazil (Flach & Dias 2020; Maciel et al. 2020).

The rough-toothed dolphin inhabits tropical, subtropical and warm-temperate waters in all three major oceans, mostly between 40°N and 35°S. This species usually occurs in oceanic waters deeper than 1,000 m, but also in shallower waters (until 30 m or less) (West et al. 2011; Jefferson 2018; Lodi & Maricato 2020). This species lives in relatively small groups, mostly ranging from 10-20 individuals, although larger groups up to 50 or more individuals and mixed groups in association with other species have been documented (Jefferson 2018). The conservation status of the rough-toothed dolphin ('least concern'; Kiszka et al. 2019) suggests that, globally, the populations are not threatened; however, considering the anthropogenic activities and disturbances in the dolphins' habitats, such as fisheries, ports, chemical and sound pollutions, marine debris and climate change, monitoring of their populations is recommended (Jefferson 2018).

In northern Rio de Janeiro State (21ºS-22ºS), past surveys on the relationship between fisheries and dolphins indicated that the rough-toothed dolphin was occasionally captured in gillnet operations practiced up to the 50 m depth contour (Di Beneditto et al. 1998; Di Beneditto 2003). In this region, it is likely that the rough-toothed dolphin occurs in deeper waters, far from the coastline and gillnet fisheries; however, inhabiting a deeper environment does not necessarily mean that the species is far from anthropogenic influences. The northern Rio de Janeiro State faced an economic boom in the 1970s, when offshore oil deposits were discovered in deep waters of the Campos Basin, a sedimentary basin that covers approximately 100,000 km^2 from 19°S to 23°S and corresponds to 70-80% of Brazilian domestic production of oil and gas (Rodriguez & Suslick 2009). These local port activities provide logistics to the oil and gas production that have been growing since then, affecting coastal areas (Zappes et al. 2016). In this context, recent assessments have indicated a considerable burden of contaminants in populations of rough-toothed dolphins along the SE Brazilian coast (Monteiro et al. 2019, 2020).

This study presents the solitary behavior of a sociable rough-toothed dolphin in a port area on northern Rio de Janeiro State, Brazil. Our purpose is to contribute to the understanding of this unusual and peculiar behavior reported in a few species worldwide.

Materials and Methods

In December 2019, we received a report from a citizen (S.R. Gomes) from Atafona village (21º37'S; 041º01'W) regarding a solitary dolphin that was wandering the Açu Superport area (21º49'S; 041º00'W) (Figure 1). A superport is a large port capable of accommodating very large ships, especially supertankers of 100,000 tons or more. The Açu Superport is a private port that started to be built in 2008 and it has been in operation since 2014. Marine traffic density maps demonstrate significant increase in ships movement since 2016 (available at: https://www.marinetraffic.com/en/ais/home/centerx:-41.0/centery:-21.8/zoom:11).

The Açu Superport is the main hub for oil and gas in Brazil, providing logistics to offshore activities in Campos Basin. The Açu Superport area encompasses 90 km^2 subdivided into two large terminals that operate 24/7. The Terminal 1 is the offshore terminal with five piers for handling iron ore and oil. The Terminal 2 is the onshore terminal installed around an internal channel with seven Private Use Terminals (PUTs). The PUTs handle solid and liquid bulk cargoes and provide logistics for oil and gas exploration activities. The internal channel of Terminal 2 has an "L" shape and two piers, north and south, protecting its entrance (Figure 1). The channel is 6.5 km long, 270 m wide and 14 m deep in the east-west section, and 120 m wide and 10 m deep in the north-south section (available at: https://portodoacu.com.br/wp-content/uploads/2018/02/Informacoes-Portuarias-T2.pdf).

In February 2020, we were introduced to four professionals (three sailors and one diver), residents from Atafona village, who work in supply boats in the Açu Superport and have personally interacted with the dolphin. They sent us mobile phone videos and agreed to provide information about the dolphin after we explained the aims of the study.

The interviewees answered the same questionnaire, with open questions about the routine in the Açu Superport, since when the solitary dolphin has been there, the dolphin's behavior, and if they swim with the dolphin, touch it or offer food to it. Each interview was conducted individually to avoid interference during the dialogue. Since the answers of the four interviewees were convergent, we considered them as a single narrative about the presence of the solitary rough-toothed dolphin in the Açu Superport, including its behavior. Only the specific reports made by the diver dealing with his underwater experience with the dolphin were highlighted from the answers.

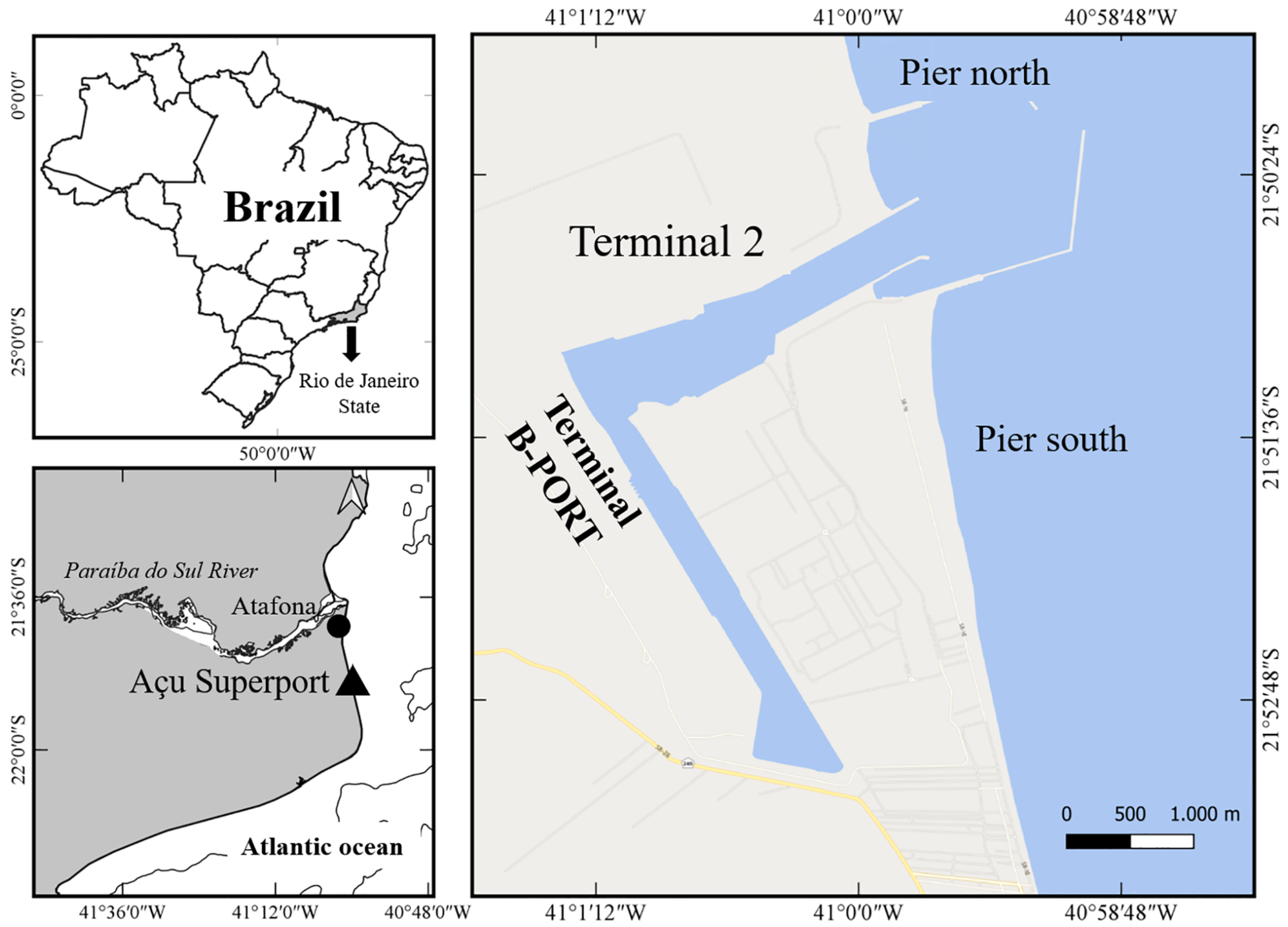

Figure 1. Location of the Açu Superport in northern Rio de Janeiro State, SE Brazil, and the Terminal 2 where the solitary rough-toothed dolphin has lived since 2017-2018.

Results and Discussion

The solitary dolphin was identified as a rough-toothed dolphin, *Steno bredanensis* (Cetartiodactyla, Delphinidae) according to mobile phone videos taken by the interviewees (Figure 2). The body features with taxonomical importance noted in the images were: i) no prominent melon and no crease between the beak and forehead, ii) forehead and sides of the head sloping smoothly onto a long and slender beak, iii) dark grey color forming a narrow cape or band along the back with white below, iv) light-colored blotches on the body, and v) white lips and lower jaw (Figure 2) (Jefferson 2018). Since young rough-toothed dolphins have a muted color pattern and generally lack the white blotches on the body (Miyazaki & Perrin 1994), we can infer that this solitary dolphin is an adult individual.

Since 2017, the four interviewees have worked regularly on supply boats at Terminal 2 of the Açu Superport. The three sailors were former fishers at Atafona village, and one of them is a professional diver. They usually stay a two-week period on board, in the port area, and come back home for a resting period of another two-week. The supply boat can be demanded both day and night, according to ships and/or tugs movement. Thus, the interviewees navigate through the internal channel of Terminal 2 and adjacent open marine waters daily.

According to the interviewees, the solitary dolphin was seen for the first time near the Açu Superport between 2017 and 2018, and it has been there until the organization of the study data (April 2020). Indeed, there are no previous videos or photos to prove by photo identification method, for instance, that it is the same dolphin from 2017-2018 until 2020. We had the same report made by four different interviewees and the literature data that describe solitary dolphins as individuals with restrict movements to a small area, with time interval of site fidelity from few weeks to months, or even decades (20-40 years) (Müller & Bossley 2002, Nunny & Simmonds 2019). Thus, we assumed that this record is probably from the same dolphin with local site fidelity over two years up to now.

The dolphin frequently swims next to the supply boat while navigating along the internal channel of Terminal 2, but it remains close to the access channel, between the piers north and south, even if the boat continues towards open sea (Figure 1). Then, the solitary dolphin returns to the Terminal 2. This route is done during daytime, but it has already been recorded at night. The dolphin frequently displays bow-riding and jumping behaviors near the supply boat (Figures 2). These aerial behaviors are common in free-ranging rough-toothed dolphins, as reported off La Gomera, Canary Islands, in a long survey (Ritter 2002).

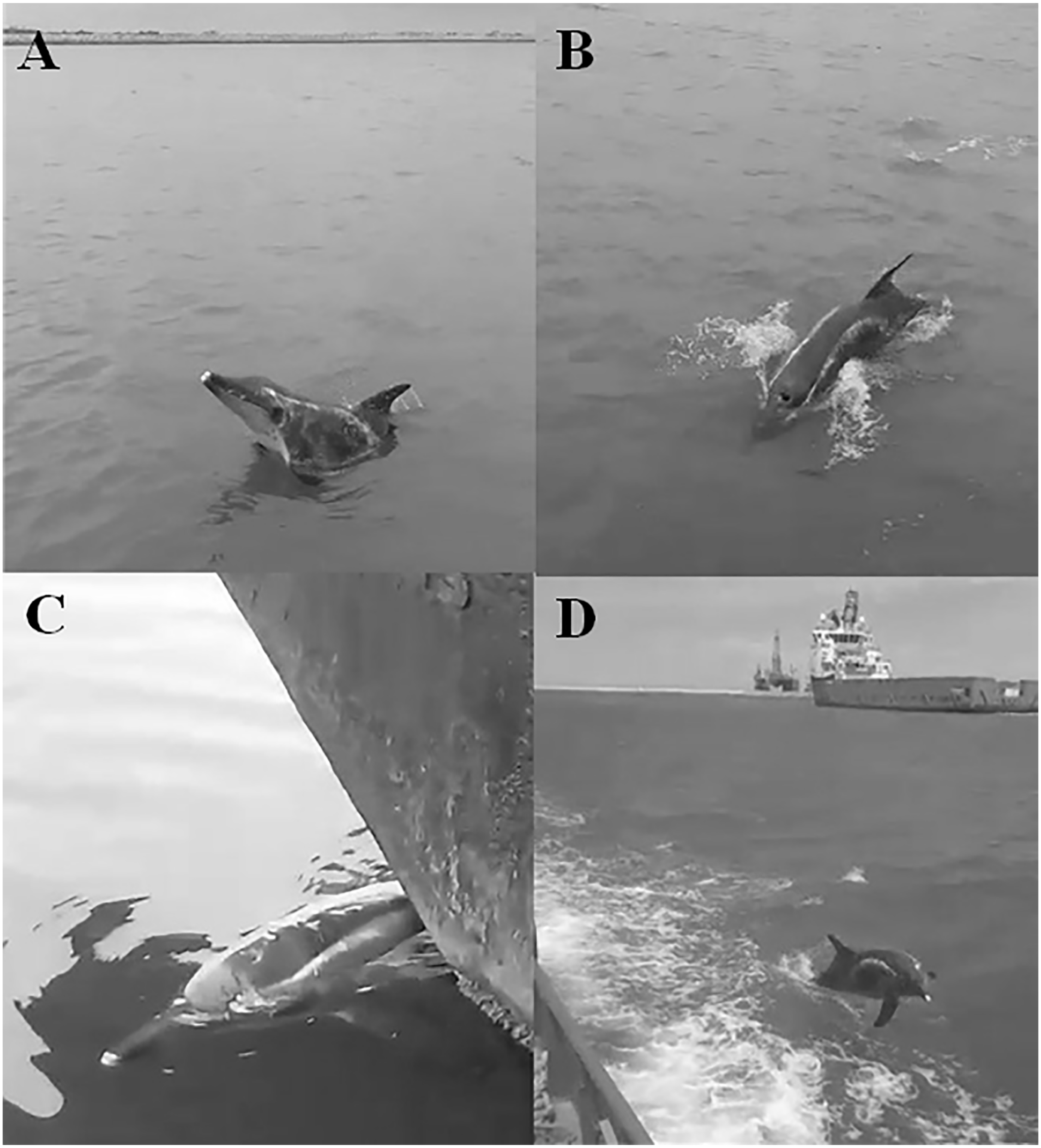

Figure 2. The solitary rough-toothed dolphin in the Açu Superport. Images available from mobile phones videos used by the interviewees in December 2019. A and B) Dolphin swimming next to the supply boat; C) Dolphin rubbing itself against a hull inside the Terminal 2; and D) Dolphin bow-riding next to the supply boat - in the background a tug and the oil platform anchored near the pier south (until February 2020).

The interviewees also reported occurrences of Guiana dolphin (*Sotalia guianensis* Van Bénéden 1864), locally known as 'boto', and franciscana (*Pontoporia blainvillei* Gervais & d'Orbigny 1844), known as 'toninha', near the Terminal 2 entrance. Since the three sailors were fishers that dealt with incidental captures of dolphins locally, their reports on Guiana dolphin and franciscana near the Açu Superport are reliable (Di Beneditto 2003; Rosa et al. 2012). They did not see interactions between these dolphins and the solitary rough-toothed dolphin. However, as dolphins' sights are not the purpose of the interviewees, it does not mean that interactions do not happen.

When the solitary dolphin is not swimming near the supply boat, it remains close to the Terminal B-PORT, located 2.6 km from the Terminal 2 entrance (Figure 1). The Terminal B-PORT is one of the PUTs, operated by *Brasil Port Logística Offshore and Estaleiro Naval Ltda* to provide logistics to the oil and gas operations and to make repairs on ships. The dolphin plays with the buoys and containment barriers that surround ships and tugs anchored for supply or repair, and sometimes it rubs itself against hulls (Figure 2). During the 2nd half of 2019, an out-of-service oil platform remained anchored in coastal waters close to pier south, at 21º50'45S; 40º59'12W (Figure 2). The solitary dolphin followed the supply boat during regular platform checking, but eventually it made the journey alone (Terminal 2-platform-Terminal 2). In February 2020, this platform was moved to open sea.

The interviewees have never swum with the solitary dolphin, touched it, or offered it food, and they were unaware of anyone who has ever done so in the Açu Superport. The diver reported that when he is underwater, the dolphin stays nearby, and sometimes it emits sounds and hits the caudal fin on divers' bodies 'just to scare, or for fun', in his own words. The diver never tried to touch the dolphin intentionally. The interviewees did not observe the solitary dolphin feeding. However, they reported plenty of fish in the Terminal 2 that can be consumed as food items.

To become a solitary dolphin, the individual undergoes many behavioral stages, named stages of sociability, as proposed by Wilke et al. (2005) (stages 1 to 4) and Nunny & Simmonds (2019) (stages 0 and 5). Both classifications of behavioral stages share the stages 1 to 4. Briefly, at stage 0 the dolphin is simply one seemed to be persistently on its own and may be seen in multiple locations. At stage 1, the dolphin establishes itself in a limited home range and may follow boats or approach fishing gears, but it does not approach people. At stage 2, the dolphin follows boats more regularly and it is engaged in investigating ropes, chains, buoys, being interested in people who enter the water, but it maintains a distance. At stage 3, the dolphin gets used to one or more people who have deliberately tried to habituate it, swimming, diving, touching and even holding it. At stage 4, the dolphin becomes a 'tourist attraction', and people come from further afield to see and swim with it. At this stage, the animal may start to exhibit dominant, aggressive and sexual behaviors. When a dolphin reaches the stage 5 (less common), it returns to live with conspecifics and ceases to be solitary. Wilke et al. (2005) suggest that some solitary dolphins only progress to stage 2 or 3, such as noted for the rough-toothed dolphin in the Açu Superport. Indeed, the study case seems to be an intermediate behavior between stages 2 and 3 because the solitary rough-toothed dolphin seems to be accustomed to the interviewees, but they do not touch the dolphin intentionally.

The known records of solitary rough-toothed dolphin were from SE Brazil. Past events have been recorded, including an unreported case made by one of the authors (Siciliano, S. personal observation), which attracted the media and human attention in Praia da Costa, Espírito Santo State (20º19'S; 040º17'W). Observations of this solitary rough-toothed dolphin lasted from September 12 to September 17, 1994, at least. It was an agile dolphin, apparently in good health condition. During the observation period, it was actively chasing fish and it was often leaping and playing with floating leaves, but not approaching humans. After a week, the dolphin left the beach. No other dolphin of this species was seen nearby during the period. This episode should be noted as the first case of a solitary rough-toothed dolphin worldwide, a stage 0 according to Nunny & Simmonds (2019).

Simultaneously to our present observations, there was a record of a solitary rough-toothed dolphin in Sepetiba Bay, southern coast of Rio de Janeiro State (Flach & Dias 2020, Maciel et al. 2020). Observations started in November 2018 and lasted until March 29, 2020, when its carcass was found floating in Angra dos Reis Bay, which is adjacent to Sepetiba Bay, according to the National Center for Research and Conservation of Aquatic Mammals - ICMBio/CMA (available at: https://www.facebook.com/ICMBioCMA/posts/853742715127036/). Therefore, these two records on solitary behavior of sociable rough-toothed dolphin in coastal waters from Rio de Janeiro State (21ºS-23ºS) were concurrent (2018-2020). The distance between the two sites (Sepetiba Bay and Açu Superport) is approximately 350-400 km. There are other similarities between both records. Considering the stages of sociability described above, the dolphin is/was at stage 2-3. Most sightings of the rough-toothed dolphin in Sepetiba Bay were in an area where fisheries (and civilian boat traffic) are restricted because of a military base (Brazilian Navy). Thus, according to Flach & Dias (2020) the lack of fisheries might increase the availability of prey species, attracting the solitary dolphin to this restricted area. The marine coastal waters around Açu Superport are also a fishing exclusion zone (Zappes et al. 2016), and the interviewees reported the high availability of fish in Terminal 2, where the dolphin usually stays. Therefore, it is reasonable to consider that food availability contributes/contributed to the dolphin's site fidelity in both areas.

According to the data, the solitary rough-toothed dolphin that lives in Açu Superport area is not disturbed locally and it is observed from a distance (usually from boats). The only physical interaction reported was a dolphin's initiative, and it was not encouraged by the professional diver. There is no need to alert the environmental authorities, mainly because the Açu Superport is a restricted access area, which prevents the dolphin's contact with people who could have agonistic attitudes against it. However, eventual risks to the dolphin locally could be oil spill, since the Açu superport is an oil production hub, boat collision and underwater noise levels.

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Authors' Contribution

Ana Paula Madeira Di Beneditto: contributed in the concept and design of the study, data collection and interpretation, and manuscript preparation.

Salvatore Siciliano: contributed in the data interpretation and critical revision.

Conflicts of Interest

The authors declare that they have no conflict of interest related to the publication of this manuscript.

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Article

Lycophytes of the Chapada das Mesas National Park, Cerrado, Maranhão, Brazil

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Abstract: Despite an increase in studies involving lycophytes and ferns in recent years, Maranhão is still one of the northeastern states with a poorly known flora, mainly due to low sampling. For Chapada das Mesas National Park, a protected conservation unit in the Cerrado maranhense with phytophysiognomies that favor floristic diversity, there are no floristic studies about lycophytes. This study aimed to conduct the floristic inventory and taxonomic study of lycophytes in Chapada das Mesas National Park. Three collection expeditions, of three days each, were made between March 2017 and June 2018. Three families of lycophytes represented by three genera and six species were identified in the study area. The most diverse family was Selaginellaceae with four species: *Selaginella conduplicata, S. erythropus* (Mart.) Spring, *S. radiata* (Aubl.) Baker and *S. simplex* Baker. The other two families, Isoëtaceae and Lycopodiaceae, had one species each, *Isoëtes* sp. and *Palhinhaea cernua* (L.) Franco & Vasc. This study also reports two new record for the Brazilian Cerrado phytogeographic domain, S. conduplicate and S. radiata, of which the first is a new record for Maranhão State. Most specimens were found in riparian forest on rocky outcrops.

Keywords: *Riparian forest; Selaginella; Taxonomy; Waterfall.*

Licófitas do Parque Nacional Chapada das Mesas, Cerrado Maranhense, Brasil

Resumo: Embora as pesquisas envolvendo o estudo de licófitas e samambaias tenham crescido nos últimos anos, o Maranhão ainda é um dos Estados do Nordeste com sua flora menos conhecida em virtude, principalmente, da baixa amostragem. O Parque Nacional (PARNA) Chapada das Mesas, apesar de tratar-se de uma unidade de conservação protegida no Cerrado maranhense e abrigar fitofisionomias que são propícias à diversidade florística, ainda não possui estudos sobre a diversidade florística de licófitas. Este estudo teve como objetivo geral fazer um inventário e estudo florístico e taxonômico de licófitas para o PARNA Chapada das Mesas. Foram realizadas três expedições para coletas de material entre março 2017 e junho 2018 com duração de três dias cada. Na área de estudo foram identificadas as três famílias de licófitas representadas por três gêneros e seis espécies. A família com maior diversidade foi Selaginellaceae com quatro espécies *Selaginella conduplicata, S. erythropus, S. radiata* e *S. simplex*. As outras duas, Isoëtaceae e Lycopodiaceae com uma espécie cada, *Isoëtes* sp., e *Palhinhaea cernua*. Nesse estudo também são relatados dois novos registros para o domínio fitogeográfico do Cerrado, *S. conduplicata* e *S. radiata*, sendo a primeira um novo registro para o Estado do Maranhão. A maioria dos espécimes foi encontrada em ambientes de matas ciliares crescendo sobre afloramento rochoso.

Palavras-chave: *Mata ciliar; Selaginella; Taxonomia; Cachoeira.*

Introduction

The class Lycopodiopsida, also known as lycophytes, is a monophyletic group characterized by its main leaves that are microphylls, sporangia with transversal dehiscence in the adaxial axil of the sporophyll and, in most cases, strobili forming at the branch apex (Tryon & Tryon 1982, Banks 2009, PPG I 2016). Among vascular plants, it is considered the most basal group (Pryer et al. 2001), with the first fossil records dating to the Paleozoic era, approximately 350–410 million years ago (Kenrick & Crane 1997).

Lycophytes are herbaceous and can be terrestrial, epiphytic, rupicolous or aquatic (Prado & Sylvestre 2010). They are widely distributed in many ecosystems of tropical, subtropical, temperate and boreal regions, which demonstrates their great adaptive success (Moran & Smith 2001). According to Kenrick & Crane (1997), the extant representatives of lycophytes belong to three families, Lycopodiaceae, Isoëtaceae and Selaginellaceae, of which the first is homosporous and the last two are heterosporous.

Lycopodiaceae species are distributed on practically all continents, especially in the neotropical region (Øllgaard 1995A). According to the classification by Øllgaard (2012), the family is divided into 16 genera and represented by terrestrial, epiphytic and rupicolous plants. These taxa are highly diverse in South America, exhibiting a variety of life forms, and are found in various habitats (Arana & Øllgaard 2012, Arana et al. 2017).

The family Isoëtaceae is monogeneric (*Isoëtes*), is distributed worldwide and comprises approximately 250 species and comprises plants that are usually submersed or emergent, including some that are seasonal with active growth during spring (Jermy 1990, Moran 2012, Troia et al. 2016). Due to their morphological simplicity (Taylor & Hickey 1992), reproductive structures, such as megaspore and microspore ornamentation (Pfeiffer 1922, Hickey 1986, Musselman 2002), as well as anatomical and leaf morphology diagnostic characters, are important in the classification of the species (Rolleri & Prada 2007).

The family Selaginellaceae is also represented by one genus, *Selaginella*, which is cosmopolitan and widely adapted, mainly in the tropics (Moran 2012, Tryon & Stolze 1994). The genus is characterized by high intraspecific variability, as well as reduced interspecific variation (Schulz et al. 2013).

Lycophytes comprise approximately 1,338 species (PPG I 2016) and represent only 1% of vascular plants (Moran 2008). In Brazil, there are 186 lycophyte species, of which 49 have been recorded in the Northeast Region, a relatively low number compared to the other regions of the country, except the Central-West region where there are only 38 species. The others Brazilian regions are better sampled, with 83 in the North, 95 in the Southeast, and 64 in the South (Flora do Brasil 2020, under construction).

Although it is one of the largest states in the Northeast Region, Maranhão is still undersampled. Local floristic studies about seedless vascular plants contain few species and have recorded only eleven species of lycophytes (e.g., Bastos & Cutrim 1999, Fernandes et al. 2007, Fernandes et al. 2010, Conceição & Rodrigues 2010, Silva et al. 2017, Santos-Silva et al. 2018, Flora do Brasil 2020, under construction). Maranhão State is situated in an ecotone area between the Amazon and Brazilian Cerrado phytogeographic domain and acts as an ecological corridor (called the Araguaia-Bananal) that connects these areas, as well as the Caatinga and coastal environment (ICMbio 2019). The Brazilian Cerrado phytogeographic domain it has high floristic diversity, including a third of the biodiversity in Brazil and around 5% in the world (Klink & Machado 2005).

The protected area Chapada das Mesas National Park, created under the decree of 12 December 2005 (BRASIL 2005), mainly has phytophysiognomic features of Brazilian Cerrado, including large formations of grassland and savanna, as well as natural ecosystems of high ecological and socio-environmental value (ICMbio 2019). Despite being established over a decade ago and its ecological relevance, there are few published works about the plant biodiversity that support the creation of a management plan for the area (Oliveira et al. 2018, Silva et al. 2018).

The phytogeographic domain in Maranhão State are under an accelerated process of destruction (Bolfe et al. 2016, Silva Junior et al. 2018) and studying the floristic diversity of the state is important since it is poorly sampled (Flora do Brasil 2020, under construction).

Thus, the objective of the present study was to inventory and provide a taxonomic treatment of the lycophyte species in Chapada das Mesas National Park, Maranhão State, Brazil.

Material and Methods

1. *Study area*

The study area, Chapada das Mesas National Park, comprises approximately 160,000 ha of Cerrado divided into two polygons, one with around 120,000 ha and another with about 40,000 ha, distributed in the municipalities of Carolina, Riachão and Estreito in southeastern Maranhão State (ICMbio 2019).

The climate is predominantly seasonal tropical, type Aw (Köppen 1948), with a dry season for 3 to 5 months and a rainy season with average rainfall ranging from 1,250 to 1,500 mm, an average annual temperature between 20ºC and 27ºC and average relative air humidity of approximately 60% (Pereira et al. 2011).

Hydrologically, the area is very rich. The main water courses are the Farinha River (norther portion) and Itapecuru River (southern portion), there are over 400 springs in the interior, and along the courses there are well-conserved gallery forests (ICMBio 2019).

The relief is characterized by the presence of sandstone and typical forms of paleokarst in sandstone (e.g., hilltop arches, small cavities, canyons, paleosinkholes, sinkholes and river resurgences), which is closely related to the evolution of the subterranean drainage and river incision (Martins et al. 2017). The soil is predominantly sandy, where there are the Brazilian Cerrado sensu stricto and *campo sujo* physiognomies. However, there are also places with richer soils, mainly on the tops of mountains that have patches of semideciduous forest (ICMBio 2019).

In addition to the natural vegetation cover typical of the Brazilian Cerrado phytogeographic domain that contains many phytophysiognomies, among which savanna and forest aspects are notable, there are also Caatinga and Amazonian species, which help characterize the high diversity of the area (ICMBio 2019). These vegetation characteristics, including the presence of well-conserved gallery forests along water courses, large patches of *cerradão* and other types of phytophysiognomies, are essential for maintaining the biodiversity in the region (Marques 2012).

2. *Data collection*

Samples of lycophytes were collected in Chapada das Mesas National Park and the surrounding area under permit number 50167-4 issued by the Sistema de Autorização e Informação em Biodiversidade do Instituto Chico Mendes de Conservação da Biodiversidade- Sisbio/ICMBio.

The species were collected during three expeditions, in March and October 2017, and June 2018, which were three days each and included the dry and rainy seasons. Collecting was based on the methods proposed by Filgueiras et al. (1994). Microenvironments in the study area were selected and explored randomly, with the goal of visiting the greatest number of microenvironments where taxa might occur. The sampling points were mapped (Figure 1).

Herbarium specimens were made base on standard techniques used for seedless vascular plants (Silva 1989, Windisch 1992). All the material was deposited in the herbarium CCAA (RBH 2019), at the Federal University of Maranhão, *Campus* Chapadinha. When available, duplicates were sent to the following institutions: MG, HBRA and BHCB (Thiers 2019).

The identification and distribution of the species and genera were based on specialized literature, such as Alston et al. (1981), Fraile et al. (1995), Øllgaard (1995 A, B), Smith (1995), Hirai & Prado (2000), Góes-Neto et al. (2016), Heringer et al. (2016), Øllgaard & Windisch (2016), Flora do Brasil 2020 (under construction), as well as revisions and neotropical floras (e.g., Tryon & Stolze 1994, Moran & Riba 1995, Mickel & Smith 2004). When needed, some species were sent to specialists to confirm the identification. The morphological study, inspection and descriptions were based only on examined material (see results).

The terminology follows Lellinger (2002). Family and genus delimitations are based on PPG I (2016). Nomenclature and authors of the species follow the International Plant Names Index (IPNI 2019) and Tropicos (2019).

Results

1. *Diversity*

Six species of lycophytes were recorded in Chapada das Mesas National Park, including three families and three genera: Selaginellaceae with 4 species, *Selaginella conduplicata* Spring, *S. erythropus* (Mart.) Spring, *S. radiata* (Aubl.) Baker and *S. simplex* Baker; Isoëtaceae, *Isoëtes* sp.; and Lycopodiaceae, *Palhinaea cernuna* (L.) Franco & Vasc.

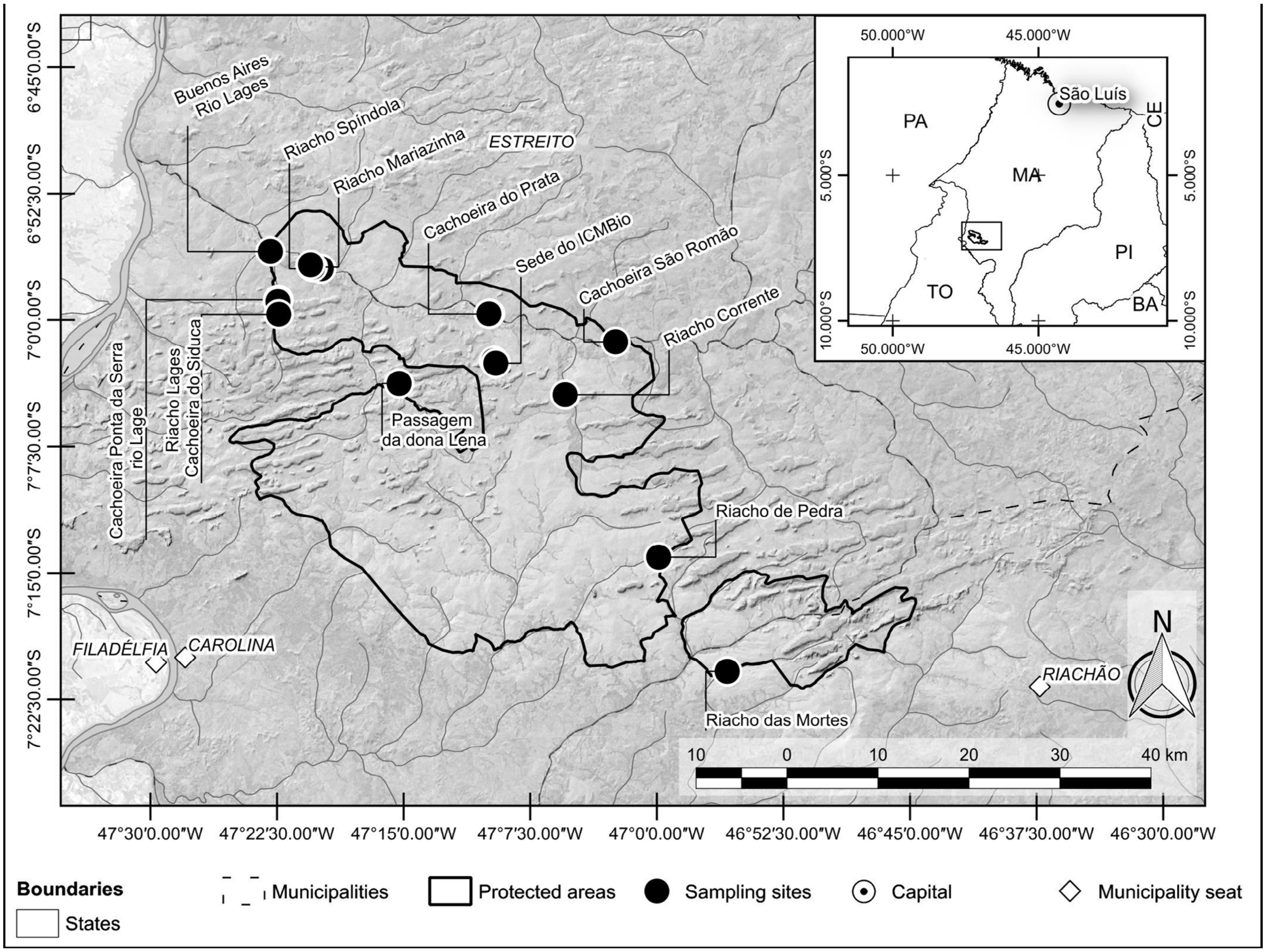

Figure 1. Collecting sites in the Chapada das Mesas National Park, Maranhão State, Brazil.

2. *Identification key to the lycophyte species in Chapada das Mesas National Park*

1. Plant homosporous; microphylls arranged in alternate whorls, not ligulate, acicular; lateral branches patent; strobili pendent at the apex of branches……………………..……………........***Palhinhaea cernua***

1'. Plant heterosporous; microphylls arranged in four rows or forming a rosette, ligulate, linear, lanceolate, ovate-lanceolate to ovate-elliptic; lateral branches not patent; strobili absent or erect at the apex of branches...**2**

2. Microphylls linear, 9.5–21.5 cm long, with four air chambers in transversal section, deposited in the form of a rosette on a corm stem type; sporangia fused in the base of the sporophyll...***Isoëtes* sp.**

2'. Microphylls not linear (< 1 cm long), without air chambers in transversal section, arranged in four rows on aerial and prostrate branches; sporangia not fused, in strobili at the apex of branches...**3**

3. Stem articulate; lateral microphylls with two denticulate auricles; base of dorsal microphylls with an auricle on the outer side; axillary microphylls with two dentate auricles with sparsely denticulate margin……………………………………………………………..........***Selaginella conduplicata***

3'. Stem not articulate; lateral microphylls without auricles; base of dorsal microphylls without auricles, rounded to slightly cuneate; axillary microphylls without auricles, truncate, rounded or sometimes subcordate, margin ciliate to serrate, rarely entire ………………...........………......**4**

4. Base of stem reddish; dorsal microphylls with ciliate margin; axillary microphylls with acuminate to attenuate apex.. ....***Selaginella erythropus***

4'. Base of stem stramineous; dorsal microphylls with denticulate-eciliate margin; axillary microphylls with acute apex.....................................**5**

5. Lateral microphylls with ciliate margin on the basal acroscopic side; dorsal microphylls with strongly aristate apex; axillary microphylls with ciliate margin at the base...***Selaginella radiata***

5'. Lateral microphylls with denticulate margin on the basal acroscopic side; dorsal microphylls with acuminate to long-acuminate apex; axillary microphylls with sparsely serrate-eciliate margin……………………...…………………...……………………………....………...***Selaginella simplex***

3. *Taxonomic treatment, distribution and comments*

Palhinhaea Franco & Vasc., Bol. Soc. Brot., sér. 2, 41: 24. 1967.

The genus is represented by 25 species (PPG I 2016). In Brazil, there are six species, which occur in all five regions politic and in the Amazon, Brazilian Cerrado, Atlantic Forest, Pampa and Pantanal phytogeographic domain. Of these, two species have been recorded in the Northeast Region and one in Maranhão.

1. Palhinhaea cernua (L.) Franco & Vasc., Bol. Soc. Brot., sér. 2, 41: 25. 1967.

Plant terrestrial or rupicolous, 47.5–187 cm long. **Stem** erect to long-scandent, flagelliform at the apex, stoloniferous, greenish to stramineous, glabrous; **lateral branches** dichotomously branched, lateral branches patent; **terminal branches** 4–5 mm wide (including microphylls); **sterile microphylls** isophyllous, microphylls arranged in alternate whorls, 3–4 mm long, acicular, gradually changing on the main axis from reflexed to patent, curved in the direction of the apex, apex cuneate, base decurrent, margin entire, glabrous or with sparse trichomes; **strobili** 3–20 × 1–2 mm, pendent at the apex of lateral branches; **sporophylls** 1.5–2 mm long, ovate-deltoid, apex long-cuspidate to acuminate, base coalescent, margin erose to laciniate; not ligulate; plant homosporous.

Specimens examined: BRAZIL, MARANHÃO, PARNA Chapada das Mesas, Carolina, Cachoeira da Ponta da Serra, Riacho Lages, 6º58'47,4" S, 47º22'25" W, 235 m, 11 de March de 2017, *L.R. Silva & M.R. Pietrobom 31* (CCAA807); idem, *L.R. Silva & M.R. Pietrobom 78* (CCAA806); idem, Riacho Corrente, 7º04'25,6" S, 47º05'26,6" W, 284 m, 13 de March de 2017, *F.C. Almeida et al. 71* (CCAA854); idem, 7º04'25,0" S, 47º05'26,2" W, 277 m, 7 de June de 2018, *F.C. Almeida et al. 75* (CCAA855).

Distribution: The species has a Pantropical distribution (Øllgaard & Windisch 2016) and occurs in Asia, Africa, the Antilles, Belize, Bolivia, Brazil, Cameroon, Costa Rica, Colombia, El Salvador, United States of America, Ecuador, Guatemala, Guinea, Honduras, Madagascar, Mexico, Nicaragua, Oceania, Paraguay, Peru, Suriname, Trinidad, and Venezuela (Øllgaard 1995B). In Brazil, it is widely distributed and occurs in all of the regions: North (Amazonas, Amapá, Pará, Rondônia, Roraima, Tocantins); Northeast (Alagoas, Bahia, Ceará, Maranhão, Paraíba, Pernambuco); Central-West (Distrito Federal, Goiás, Mato Grosso do Sul, Mato Grosso); Southeast (Espírito Santo, Minas Gerais, Rio de Janeiro, São Paulo); and South (Paraná, Rio Grande do Sul, Santa Catarina).

Comments: *Palhinhaea cernua* is characterized by its dichotomously branched, patent lateral branches and pendent strobili. The strobili have been described as being 4–20 mm long and 2.5–3 mm wide (Øllgaard 1995A, Øllgaard & Windisch 2016); however, in the samples examined from Chapada das Mesas National Park variations starting at 3 mm long and 1–2 mm wide were recorded.

The most similar species to *P. cernua* that also occurs in the Northeast Region is *P. camporum* (B. Øllg. & P.G. Windisch) Holub, since it has an erect, branched stem and pendent strobili. However, *P. cernua* has recurved distal branches and a more flexible stem, while *P. camporum* has non-recurved distal branches and a rigid stem (Øllgaard & Windisch 2016).

In the study area, *P. cernua* was collected in an open environment, with a lot of or little sun, on soil and rock near a stream and waterfall.

Isoëtes L., Sp. Pl. 2: 1100. 1753.

The genus is represented by around 250 species (PPG I 2016). In Brazil, 28 species have been recorded, which are distributed in the five regions in the Amazon, Brazilian Cerrado, Altantic Forest, Pampa and Pantanal phytogeographic domain. From these 28 species, three have been recorded for the Northeast Region, *Isoëtes gigantea* U. Weber, *I. luetzelburgii* U. Weber, *I. panamensis* Maxon & C. V. Morton, the latter one also for Maranhão.

1. Isoëtes sp.
(Figure 2F)

Plant amphibious, 10.2–22.3 cm long. **Stem** globose, corm type, 2–3 lobate; **microphylls** imbricate, forming a rosette, with four air chambers in transversal section, deposited in the form of a rosette, 9.5–21.5 cm long, linear, erect, laminated, subula distal and with wings restricted to the proximal portion, septum with stellate, greenish cells; strobili absent; **sporangia** fused in the base of the sporophyll; **megaspores** trilete, distal and proximal surfaces baculate, whitish; plant heterosporous.

Specimens examined: BRAZIL, MARANHÃO, PARNA Chapada das Mesas, Carolina, Cachoeira do Prata, Rio Farinha, 6º59’36,9” S, 47º9’58,5” W, 198 m, 12 March 2017, *L.R. Silva & M.R. Pietrobom 45* (CCAA808).

Comments: The material collected of *Isoëtes* is sterile, but 4 or 5 megaspores were found loose in the substrate near the stem. Thus, even by a specialist, it is not possible to determine the species (personal communication by Dr. Jovani Bernardino de Sousa Pereira) since the taxonomy of the group is mainly based on reproductive structures, such as sporangia and spores (Hickey 1986). The specimen analyzed is similar to *Isoëtes panamensis* Maxon & C.V.Mort in microphyll and megaspore characters, such as the presence of hyaline wings on the proximal portion of the microphyll, triquetrous subula and baculate surface of the megaspore. However, additional collections are needed to confirm the species.

In the study area, it was collected in an open, sunny environment near a waterfall, growing as an amphibious plant where there is seasonal flooding.

Selaginella P. Beauv. Mag. Encycl. 9(5): 478. 1804.

The genus is represented by around 700 species (PPG I 2016). In Brazil, 96 species have been recorded, which are distributed in the five regions of the country and in the Amazon, Caatinga, Brazilian Cerrado, Atlantic Forest and Pantanal phytogeographic domain. Of these, 22 species are cited for the Northeast region and four are cited for Maranhão.

1. *Selaginella conduplicata* Spring, Fl. Bras. 1(2): 129. 1840.
(Figures 2A; 3A–C)

Plant terrestrial or rupicolous, 10–130 mm long. **Stem** articulate, 2–4(–5)–pinnate, not flagelliform, repent to erect, not stoloniferous, stramineous at the base, glabrous, distal branches 3–4(–5) mm wide (including microphyll); **dorsal rhizophores** throughout the extension of the decumbent stem, stramineous; **microphylls** not overlapping before the first bifurcation, heterophyllous; microphylls not linear, without air chambers in transversal section, microphylls arranged in four rows on aerial and prostrate branches**; lateral microphylls** arranged in two rows, 2–3(–4) mm long, lanceolate, ascending, apex acute, base with two denticulate auricles, smaller auricle on the external acroscopic margin widely denticulate, whitish, basioscopic margin entire to rarely widely denticulate in the direction of the apex, basioscopically greenish; **dorsal microphylls** arranged in two rows, 1–2.5(–3) mm long, ovate-lanceolate, asymmetric, apex long-acute to acuminate, base with one denticulate auricle on the external side, margin sparsely denticulate, whitish; **axillary microphylls** 1.5–2.5 mm long, lanceolate, apex acute, base with two long, dentate auricles, margin sparsely denticulate, whitish; **strobili** erect at the apex of branches, 3–15 mm long; **sporangia** not fused, in strobili at the apex of branches; **megasporophylls** 1(2), at the base of the strobilus; **megaspores** white; plant heterosporous.

Specimens examined: BRAZIL, MARANHÃO, PARNA Chapada das Mesas, Carolina, Cachoeira do Prata, Rio Farinha, 6°59’36,9” S, 47°9’58,5” W, 198 m, 12 March 2017, *L.R. Silva & M.R. Pietrobom 40* (CCAA820); idem, *L.R. Silva & M.R. Pietrobom 43* (CCAA814); idem, 6°59’37,4” S, 47°09’58,0” W, 194 m, 29 October 2017, *L.R. Silva & F.C. Almeida 84* (CCAA826); idem, 6º59’37” S, 47º09’57” W, 197 m, 6 June 2018, *F.C. Almeida et al. 03* (CCAA852); idem, 6º59’36,7” S, 47º9’53,1” W, 210 m, *F.C. Almeida et al. 29* (CCAA853).

Distribution: This species has a South American distribution, including Brazil, Guyana, French Guiana, Suriname, Colombia, and Peru (Alston et al. 1981, Smith 1995). In Brazil, it is cited for the North (Acre, Amapá, Amazonas, Pará, Roraima), Northeast (Ceará, Pernambuco), and Central-West (Mato Grosso) regions (Paula-Zárate 2005, Silva 2014). This is the first record of *Selaginella conduplicata* for the Brazilian Cerrado and the state of Maranhão.

Comments: *Selaginella conduplicata* differs from the other species collected in the study area by its articulate stem, microphylls with auricles, and presence of one megasporangium (rarely two) on the strobili.

In the study area, it was collected near a waterfall, in an open to partially shaded environment, and was rupicolous or sometimes terrestrial.

2. *Selaginella erythropus* (Mart.) Spring, Fl. Bras. 1(2): 125. 1840.
(Figures 2B; 3D–F)

Plant terrestrial or rupicolous, 15–170 mm long. **Stem** not articulate, 2–3-pinnate, flagelliform, erect, stoloniferous, reddish

Figure 2. Habitat of the lycophytes in Chapada das Mesas National Park. A. *Selaginella conduplicata*. B. *Selaginella erythropus*. C. *Selaginella radiata*. D. *Selaginella simplex*. E. *Isoëtes sp*. F. *Palhinhaea cernua*. Photos: Laryssa Reis.

at the base, glabrous, distal branches 1.5–2 mm wide (including microphylls); **ventral rhizophores** present on the lower half of the erect stem, reddish; **microphylls** overlapping before the first bifurcation, isophyllous at the base, heterophyllous above the first bifurcation; microphylls not linear, without air chambers in transversal section, microphylls arranged in four rows on aerial branches; **lateral microphylls** arranged in two rows, 1.8–2 mm long, lanceolate to ovate-lanceolate, slightly falcate, slightly ascending at the apex, apex acuminate to attenuate, base truncate, slightly rounded acroscopically, not auriculate, acroscopic and basioscopic margins ciliate to serrate, rarely entire, slightly hyaline; **dorsal microphylls** arranged in two rows, 1–1.5 mm long, ovate-lanceolate, symmetric, apex long-aristate to long-acuminate, base rounded, margin ciliate to long-dentate, hyaline to slightly hyaline; **axillary microphylls** (1.2–)1.8–2 mm long, similar to lateral microphylls, apex acuminate, base rounded or sometimes slightly truncate on the acroscopic or basioscopic side, not auriculate, margin ciliate to denticulate, serrate toward the apex, greenish; **strobili** erect at the apex of branches, (1–)2–5(–6) mm long; **sporangia** not fused, in strobili at the apex of branches; **megasporophylls** ventral, throughout the extension of the strobilus; **megaspores** white; plant heterosporous.

Specimens examined: BRAZIL, MARANHÃO, PARNA Chapada das Mesas, Carolina, Cachoeira do Prata, Rio Farinha, 6°59’36,9” S, 47°9’58,5” W, 198 m, 12 March 2017, *L.R. Silva & M.R. Pietrobom 39* (CCAA819); idem, 6°59’41,3” S, 47°09’57,4” W, 213 m, *L.R. Silva & M.R. Pietrobom 37* (CCAA818); idem, 6°59’37,4” S, 47°09’58,0” W, 194 m, 29 October 2017, *L.R. Silva & F.C. Almeida 90* (CCAA823); idem, *L.R. Silva & F.C. Almeida 91* (CCAA825); idem, 6°59’37,3” S, 47°09’58,0” W, *L.R. Silva & F.C. Almeida 100* (CCAA824); idem, 6º59’37” S, 47º09’57” W, 197 m, 6 June 2018, *F.C. Almeida et al. 08* (CCAA851); *F.C. Almeida et al. 09* (CCAA859); *F.C. Almeida et al. 14* (CCAA846); idem, Cachoeira São Romão, 7°01’15,4” S, 47°02’28,2” W, 241 m, 31 October 2017, *L.R. Silva & F.C. Almeida 183* (CCAA822); idem, 7º01’17,1” S, 47º02’27,1” W, 256 m, 7 June 2018, *F.C. Almeida et al. 65* (CCAA847); idem, Cachoeira Ponta da Serra, Riacho Lages, 6°58’47,4” S, 47°22’25” W, 235 m, 11 March 2017, *L.R. Silva & M.R. Pietrobom 24* (CCAA817); idem, *L.R. Silva & M.R. Pietrobom 29* (CCAA816); idem, Estreito, Cachoeira do Prata, Rio Farinha, 6º59’36,7” S, 47º9’53,1” W, 210 m, 6 June 2018, *F.C. Almeida et al. 26* (CCAA849); idem, *F.C. Almeida et al. 28* (CCAA848).

Distribution: This species has a neotropical distribution, including Costa Rica, Colombia, Ecuador, Peru, Bolivia, and Brazil (Fraile et al. 1995). For Brazil, it is cited for the North (Pará, Tocantins, according to Góes-Neto 2016), Northeast (Bahia, Ceará, Maranhão, Paraíba, Piauí, Rio Grande do Norte), Central-West (Distrito Federal, Goiás, Mato Grosso, Mato Grosso do Sul) and Southeast (Minas Gerais, Rio de Janeiro) regions.

Comments: The main characteristics of *Selaginella erythropus* are the reddish stem and overlapping microphylls before the first bifurcation (Fraile et al. 1995). Among the material analyzed, on some specimens there was a stolon emerging at the apex of the branches; however, according to Fraile et al. (1995), the stolons of *S. erythropus* are restricted to the base of the branches.

In the study area, this species was collected near a waterfall and margin of a river, in an open or sometimes partially shaded environment, mostly as a rupicolous plant.

Figure 3. A-C. *Selaginella conduplicata* (F.C. Almeida et al. 03 (CCAA852)). A. Lateral microphyll. B. Dorsal microphyll. C. Axillary microphyll. D-F. *Selaginella erythropus* (L.R. Silva & M.R. Pietrobom 39 (CCAA819)). D. Lateral microphyll. E. Dorsal microphyll. F. Axillary microphyll. Photos: Samuel Vieira Brito.

3. *Selaginella radiata* (Aubl.) Spring, Bull. Acad. Roy. Sci. Bruxelles 10: 143, no. 54. 1843.
(Figures 2C; 4A–C)

Plant terrestrial or rupicolous, 15–205 mm long. **Stem** not articulate, 2–3-pinnate, not flagelliform, erect, stoloniferous, stramineous, glabrous, distal branches 3–10 mm wide (including microphylls); **rhizophores** abaxial, only at the base of the main stem, stramineous; **microphylls** not overlapping before the first bifurcation, anisophyllous; microphylls not linear, without air chambers in transversal section, microphylls arranged in four rows on aerial branches; **lateral microphylls** arranged in two rows, (1.2–)2–2.8 mm long, ovate-lanceolate, ascending, apex acute, base cordate, not auriculate, basal acroscopic margin strongly ciliate and serrate toward the apex, basioscopic margin slightly serrate, greenish; **dorsal microphylls** arranged in two rows, (1–)1.5–2 mm long, ovate-elliptic, asymmetric, apex strongly aristate, base slightly cuneate, margin denticulate, denticles larger at the base on the acroscopic side, hyaline; **axillary microphylls** (1.3–)1.5–2(–2.1) mm long, lanceolate, apex acute, base truncate, not auriculate, margin ciliate in the proximal region and slightly serrate toward the apex, hyaline at the base and greenish toward the apex; **strobili** erect at the apex of branches, 2–6 mm long; **sporangia** not fused, in strobili at the apex of branches; **megasporophylls** 2(–4), at the base of the strobilus; **megaspores** whitish; plant heterosporous.

Specimens examined: BRAZIL, MARANHÃO, PARNA Chapada das Mesas, Estreito, Cachoeira do Prata, Rio Farinha, 6°59’36,7” S, 47°9’53,1” W, 210 m, 12 March 2017, *L.R. Silva & M.R. Pietrobom 47* (CCAA809); idem, Carolina, Cachoeira São Romão, 7°1’17,2” S, 47°2’27,8” W, 258 m, 13 March 2017, *L.R. Silva & M.R. Pietrobom 55* (CCAA815); idem, *L.R. Silva & M.R. Pietrobom 59* (CCAA811); idem Cachoeira São Romão, Rio Farinhas, 7º01’17,1” S, 47º02’27,1” W, 256 m, 7 June 2018, *F.C. Almeida et al. 47* (CCAA839); idem, Cachoeira Ponta da Serra, Rio Lages, 6°58’47,4” S, 47°22’25” W, 235 m, 11 March 2017, *L.R. Silva & M.R. Pietrobom 22* (CCAA810); idem, 6°58’47,1” S, 47°22’25,5” W, 238 m, 8 June 2018, *F.C. Almeida et al. 114* (CCAA840); idem, Pousada Mansinha, riacho da Mansinha, 7°07’36,5” S, 47°25’18,2” W, 286 m, 8 June 2018 *F.C. Almeida et al. 120* (CCAA842); idem, *F.C. Almeida et al. 126* (CCAA841).

Distribution: This species is distributed in South America, including Brazil, Bolivia, Colombia, Ecuador, Guyana, French Guiana, Peru, Suriname and Venezuela (Alston et al. 1981). In Brazil, it has been recorded in the North (Amapá, Amazonas, Pará, Rondônia), Northeast (Bahia, Maranhão, Piauí), and Central-West (Mato Grosso) regions (Góes-Neto et al. 2016). This is the first record of *Selaginella radiata* for the Brazilian Cerrado.

Comments: *Selaginella radiata* is characterized by the base of the lateral and axillary microphylls strongly ciliate, as well as strongly aristate dorsal microphylls. *Selaginella decomposita* Spring is similar to *S. radiata* because of the non-articulate stem, non-auriculate microphylls and aristate dorsal microphylls. However, *S. decomposita* differs from *S. radiata* by the denticulate lateral and axillary microphyll bases and acuminate to slightly aristate dorsal microphylls (Hirai & Prado 2000, Heringer et al. 2016).

In the study area, *S. radiata* was collected near a waterfall and source of streams, in an open environment and less often in the shade, as a rupicolous and rarely terrestrial plant on sandy slopes.

4. ***Selaginella simplex*** Baker, J. Bot. 23: 293, no. 304. 1885.

(Figures 2D; 4D–F)

Plant terrestrial or rupicolous, 10–28 mm long. **Stem** not articulate, 1–2 pinnate, not flagelliform, suberect, not stoloniferous, stramineous, glabrous, distal branches 1.8–2 mm wide (including microphylls); **rhizophores** adaxial, restricted to the basal part of the stem, stramineous; **microphylls** not overlapping before the first bifurcation, heterophyllous; microphylls not linear, without air chambers in transversal section, microphylls arranged in four rows on aerial branches; **lateral microphylls** arranged in two rows, 1.2–2 mm long, ovate to ovate-elliptic, ascending, apex acute, base rounded, not auriculate, acroscopic margin denticulate at the base and serrate toward the apex, greenish, basioscopic margin serrate on the apical half; **dorsal microphylls** arranged in two rows, 1–1.2 mm long, ovate-lanceolate to ovate-elliptic, asymmetric, apex acuminate to long-acuminate, base rounded to slightly cuneate, margin denticulate, greenish; **axillary microphylls** 1.2–2 mm long, ovate-elliptic, apex acute, base rounded to subcordate, not auriculate, margin sparsely serrate, greenish; **strobili** erect at the apex of branches, (2–)3–10(–13) mm long; **megasporophylls** numerous, ventral at the base of the strobilus; **sporangia** not fused, in strobili at the apex of branches; **megaspores** yellow; plant heterosporous.

Specimens examined: BRAZIL, MARANHÃO, PARNA Chapada das Mesas, Estreito, Cachoeira do Prata, Rio Farinha, ca. 6º59’36,7” S, 47º9’53,1” W, 210 m, 6 June 2018, *F.C. Almeida et al. 25* (CCAA845); idem, *F.C. Almeida et al. 27* (CCAA843); idem, *F.C. Almeida et al. 30* (CCAA844); idem, 12 March 2017, *L.R. Silva & M.R. Pietrobom 46* (CCAA813); idem, *L.R. Silva & M.R. Pietrobom 48* (CCAA812); idem, Carolina, Cachoeira São Romão, 7º1’17,2” S, 47º2’27,8” W, 258 m, 13 March 2017, *L.R. Silva & M.R. Pietrobom 58* (CCAA821).

Distribution: This species has a neotropical distribution, including Brazil, Bolivia, Costa Rica, Mexico, Trinidad and Tobago, and Venezuela (Alston et al. 1981, Fraile et al. 1995). In Brazil, it occurs in the North (Pará, Tocantins), Northeast (Bahia, Ceará, Maranhão Piauí, Pernambuco, Sergipe), and Central-West (Goiás, Mato Grosso) regions (Góes-Neto et al. 2016).

Comments: *Selaginella simplex* is similar to *S. minima* because of the lateral microphylls with a rounded base and acute apex. However, it has greenish microphyll margins, dorsal microphylls with a rounded to slightly cuneate base and a yellow megaspore, while *S. minima* has hyaline microphyll margins, dorsal microphylls with an auricle on the external side and a white megaspore (Fraile et al. 1995). Both *Selaginella simplex* and *S. minima* Baker are neotropical, but *S. minima* occurs in Argentina, Guyana, French Guiana, Honduras, Nicaragua and Panama, where *S. simplex* has not been recorded. In Brazil, both species occur in the North and Central-West regions, while only *S. simplex* occurs in the Northeast Region (Alston et al. 1981, Fraile et al. 1995, Flora do Brasil 2020, under construction).

In the study area, *S. simplex* was collected as a rupicolous plant, both in open and shaded environments. Some individuals grow with leafy liverworts.

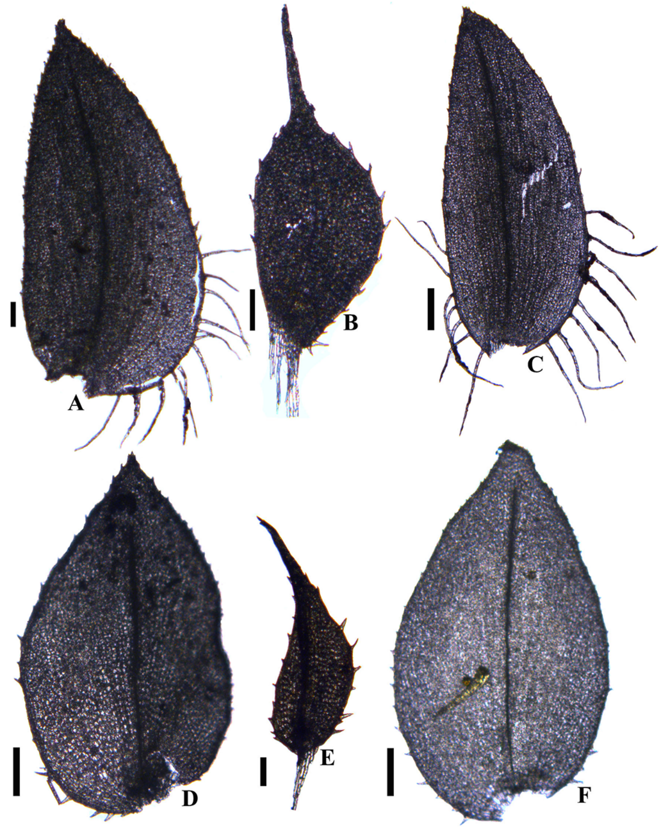

Figure 4. A-C. *Selaginella radiata* (L.R. Silva & M.R. Pietrobom 47 (CCAA809)). A. Lateral microphyll. B. Dorsal microphyll. C. Axillary microphyll. D-F. *Selaginella simplex* (F.C. Almeida et al. 25 (CCAA845)). D. Lateral microphyll. E. Dorsal microphyll. F. Axillary microphyll. Photos: Samuel Vieira Brito.

Discussion

This inventory has around 22% of the species recorded for the Brazilian Cerrado (Flora do Brasil 2020 under construction). The diversity of lycophytes listed in this work (six species) is greater in number of families and species than recorded by Bastos & Cutrim (1999), Fernandes et al. (2010), Conceição & Rodrigues (2010), Silva et al. (2017) Santos-Silva et al. (2018), which cite between one or three species, in the Lycopodiaceae and Selaginellaceae. The other published studies conducted in the Maranhão state did not record lycophytes (e.g. Fernandes et al. 2007, Conceição & Ruggieri 2010, Conceição et al. 2015, Santos-Silva et al. 2019).

This study reports an increase in the distribution of two species in the Brazilian Cerrado, *S. conduplicata* and *S. radiata* of which the first species is a new record for Maranhão State. Starting in 2015, there has been an increase in the number of studies about the Brazilian Cerrado, which has resulted in an increase in the number of lycophyte and fern species known for the phytogeographic domain (see BGF 2018). However, the known diversity for Maranhão State is still low. It is thought that the diversity of the state is greater than estimated, since it is a transition area between phytogeographic domain (mainly the Brazilian Cerrado and Amazon). Thus, there should be more and systematic sampling of the environments in this region.

Most of the species cataloged in this study are widely distributed in nearly all phytogeographic domain, such as *Palhinhaea cernua* (Amazon, Brazilian Cerrado*,* Atlantic Forest, as well as mangrove ecosystems), *Selaginella erythropus* (Caatinga, Brazilian Cerrado, Atlantic Forest,), *S. simplex* (Amazon, Caatinga, Brazilian Cerrado) (Bastos & Cutrim 1999, Azevedo & Silva 2001, Pietrobom & Barros 2003, Fernandes et al. 2007, Maciel et al. 2007, Conceição & Rodrigues 2010, Fernandes et al. 2010, Prado & Sylvestre 2010, Barros 2013, Santiago et al. 2014, Farias et al. 2017, Prado & Sylvestre 2010, Silvestre et al. 2019). The exception is *S. conduplicata* that, until now, was cited to Amazonia and Atlantic Forest in Northeastern (Ceará e Pernambuco) (Silva 2014). However, in this study it was collected in Brazilian Cerrado.

In the study area, the phytophysiognomy with the greatest diversity of species was riparian forest. According to Lehn et al. (2018), this environment is typical of the Brazilian Cerrado phytogeographic domain and favors the establishment of these species. According to Silvestre (2018), independent areas of humid forest, characteristic of transition areas, are points of species exchange, which is corroborated by the distribution of lycophytes and ferns in the region.

The Brazilian Cerrado in the Northeast Region contains ecotones, which is reflected in the floristic diversity of these areas (Walter 2006) that exhibit heterogeneous vegetation, such as gallery forest or riparian forest, and rocky outcrop vegetation (Eiten 1994). In a study of the vascular flora by Mendonça et al. (2008), it was estimated that the phytogeographic domain of the Brazilian Cerrado contains 390 taxa of seedless vascular plants in its diverse phytophysiognomies. Of this total, a little more than half occur in forest formations, and less than 8% occur in rocky environments and on the edges of streams and rivers, where nearly all of the taxa listed here occur. Further, compared to the present study, pioneering works about this Brazilian Cerrado that treat the floristic composition of lycophytes and ferns (e.g., Simabukuro et al. 1994, Forsthofer & Athayde Filho 2012, Pallos et al. 2016) cite a lower diversity of lycophytes and have only two species in common: *Selaginella erythropus* and *Palhinhaea cernua.*

Therefore, given the relevance of the data presented here, the importance of this study in relation to the Brazilian Cerrado and Chapada das Mesas National Park is evident, mainly because it increases what is known about the floristics and taxonomy of lycophytes from the Brazilian Cerrado phytogeographic domain, contributing to the biodiversity knowledge of this Protection area, and, in the future, possibly allowing appropriate conservation strategies. It is important to emphasize that the Chapada das Mesas National Park comprises half (six species) of lycophyte's diversity recorded for the Maranhão state (twelve species including the results of this study). The other six species were not found in the Chapada das Mesas National Park during our survey: *Lycopodiella geometra* B.Øllg. & P.G.Windisch, *Pseudolycopodiella carnosa* (Silveira) Holub, *P. caroliniana* (L.) Holub, *P. meridionalis* (Underw. & Loyd) Holub, *Selaginella flagellata* Spring, *S. marginata* (Humb. & Bonpl. ex Willd.) Spring (Fernandes et al. 2010, Conceição & Rodrigues 2010, Santos-Silva et al. 2018, Flora do Brasil 2020, under construction). In addition, this protected area houses one species which had never been registered previously for the state, *Selaginella conduplicata*, and other species and genus possessing a restrict geographic distribution, such as: *S. radiata* and *Isoëtes*.

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Author Contributions

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Conflicts of Interest

The authors declarethat they have no conflict of interest related to the publication of this manuscript.

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Short Communication

Sympatry between species of *Juliomys* (Rodentia: Sigmodontinae) along an altitudinal gradient in the Serra da Bocaina National Park

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Abstract: The altitudinal gradient found in the Atlantic Forest may play a role in establishing sympatry areas between congeneric species of sigmodontine rodents. For some genera with cryptic species, the elucidation of spatial patterns of distribution may be useful to understand reproductive isolation and speciation patterns, as well as spatial and temporal factors determining the boundaries of species' geographical distributions. Here we evaluated the occurrence of sympatry between *Juliomys* species along an altitudinal gradient in the Serra da Bocaina National Park (SBNP). Additionally, we review the occurrence of *J. rimofrons* and add new records of species occurrence in the SBNP, aided by karyological and molecular data. The study was carried out at four sites in the SBNP, which ranged between 770 and 1,200 m a.s.l. We captured 24 *Juliomys* specimens. Six out of 24 specimens were collected and karyotyped. For *J. pictipes*, the karyotype had a 2n of 36 and a FN of 34, and for *J. ossitenuis* 2n of 20 and FN of 36. Seventeen out of 24 *Juliomys* specimens were used in the cytochrome b phylogeny: 12 specimens grouped with *J. pictipes* and five with *J. ossitenuis*. Additionally, a specimen previously identified as *J. rimofrons* (MN 77793) clustered together with *J. pictipes*. *Juliomys pictipes* and *J. ossitenuis* were found in sympatry in two out of four sites in altitudes higher than 1,000 m a.s.l.. Our karyological and molecular data provided the detection of two Juliomys species in the SBNP for the first time (*J. pictipes* and *J. ossitenuis*) and disregarded the previous record of *J. rimofrons*. Our record of sympatry between *J. pictipes* and *J. ossitenuis* represents the fifth known record of sympatry between these species up to date.
Keywords: *Atlantic Forest; karyotype; mammals; phylogeny; Protected Area.*

Simpatria entre espécies de *Juliomys* (Rodentia: Sigmodontinae) ao longo de um gradiente altitudinal no Parque Nacional Serra da Bocaina

Resumo: O gradiente altitudinal encontrado na Mata Atlântica pode desempenhar um papel no estabelecimento de áreas de simpatria entre espécies congêneres de roedores sigmodontíneos. Para alguns gêneros com espécies crípticas, a elucidação de padrões espaciais de distribuição pode ser útil para entender os padrões de isolamento reprodutivo e de especiação, bem como fatores espaciais e temporais que determinam os limites das distribuições geográficas das espécies. Aqui, avaliamos a ocorrência de simpatria entre as espécies de *Juliomys* ao longo de um gradiente altitudinal no Parque Nacional da Serra da Bocaina (SBNP). Além disso, revisamos a ocorrência de *J. rimofrons* e adicionamos novos registros de ocorrência de espécies no SBNP, auxiliados por dados cariológicos e moleculares. O estudo foi realizado em quatro locais no SBNP, que variaram entre 770 e 1.200 m a.n.m.. Foram capturados 24 espécimes de *Juliomys*. Seis dos 24 espécimes foram coletados e cariotipados. Para *J. pictipes*, o cariótipo tinha 2n de 36 e um NF de 34, e para *J. ossitenuis* 2n de 20 e NF de 36. Dezessete dos 24 espécimes de *Juliomys* foram utilizados na filogenia do citocromo b: 12 espécimes agruparam com *J pictipes* e cinco com *J. ossitenuis*.

Além disso, um espécime previamente identificado como *J. rimofrons* (MN 77793) se agrupou com *J. pictipes*. *Juliomys pictipes* e *J. ossitenuis* foram encontrados em simpatria em dois dos quatro locais em altitudes superiores a 1.000 m a.n.m.. Nossos dados cariológicos e moleculares forneceram a detecção de duas espécies de *Juliomys* no SBNP pela primeira vez (*J. pictipes* e *J. ossitenuis*) e desconsideraram o registro anterior de *J. rimofrons*. Nosso registro de simpatria entre *J. pictipes* e *J. ossitenuis* representa o quinto registro de simpatria entre essas espécies conhecido até o momento.

Palavras-chave: *Cariótipo**;** Filogenia; Mamíferos; Mata Atlântica; Unidade de Conservação.*

Introduction

Juliomys encompasses Neotropical arboreal rodent species endemic to the southeastern Atlantic Forest of Brazil, with its northernmost distribution limited by the Doce River, in the state of Minas Gerais (González et al. 2015). The genus was described by González (2000) to allocate *Juliomys pictipes* (Osgood, 1933), originally described almost 70 years earlier as *Thomasomys pictipes*, later relocated to the genus *Wilfredomys* by Musser & Carleton (1993). Thenceforth, three additional species were described for the genus: *Juliomys rimofrons* Oliveira & Bonvicino, 2002, *Juliomys ossitenuis* Costa, Pavan, Leite & Fagundes, 2007, and more recently *Juliomys ximenezi* Christoff, Vieira, Oliveira, Gonçalves, Valiati & Tomasi, 2016 (Christoff et al. 2016). Recently, *Juliomys* was formally included in the tribe Wiedomyini Reig, 1980, which also includes *Wiedomys*, *Wilfredomys*, and *Phaenomys* (Gonçalves et al. 2020). All living species of *Juliomys* are associated with well-preserved voucher specimens, cytochrome b sequences and unique karyotypes, which facilitate species diagnoses (D'Elía et al. 2007). However, *Juliomys* includes cryptic species, not easily recognized in the field by their external morphological characters (Christoff et al. 2016). The few existing studies that differentiated species through external morphological characters found different morphological patterns in different localities, suggesting possible geographical variation in some of the characters commonly used (Pavan & Leite 2011, Aguieiras et al. 2013).

Some congeneric species of Neotropical rodents may occur in sympatry (i.e., two species occupying the same locality) or even in syntopy (i.e., two species occupying the same locality at the same time) (Gannon 1998, Gonçalves & Oliveira 2004, Cordeiro-Estrela et al. 2008, Mallet et al. 2009, Delciellos et al. 2018a). Studies on these species may be useful to better understand reproductive isolation and speciation patterns (Noor 1999), as well as ecological processes such as spatial (e.g., microhabitat selection) and temporal (e.g., divergence time) factors determining the boundaries of species' geographical distributions (Laiolo et al. 2017). *Juliomys pictipes*, the most widespread species of the genus (González et al. 2015), may present some overlapping with the known geographic distribution area of all other species (González et al. 2015, Christoff et al. 2016), but only four sympatric occurrences between *J. pictipes* and *J. ossitenuis* have been recorded so far. Sympatry between these two species was recorded in the municipalities of Caucaia do Alto (Morro Grande Forest Reserve), Mulheres, and Museros in the state of São Paulo (800 - 1000 m a.s.l.; Costa et al. 2007, Pavan & Leite 2011), and in the municipality of Teresópolis (Serra dos Órgãos National Park), in the state of Rio de Janeiro (1,200 m a.s.l.; Aguieiras et al. 2013). These records suggest that sympatry areas between these two species may occur at altitudes above 800 m a.s.l., as all localities of *J. ossitenuis* known so far are at higher altitudes (> 800 m), while *J. pictipes* are recorded in a broader altitudinal range (Costa et al. 2007, Pardiñas et al. 2008, Cerboncini et al. 2014).

Here we evaluated the occurrence of sympatry between *Juliomys* species along an altitudinal gradient in the Serra da Bocaina National Park. Additionally, we review the occurrence of *J. rimofrons* and add new records of species occurrence in the park, aided by karyological and molecular data.

Material and Methods

1. Study area

The Serra da Bocaina National Park (SBNP) is one of the few remaining large fragments of Atlantic Forest, and has an altitudinal gradient that ranges from 0 to 2,088 m a.s.l.. The first study regarding mammal species that occur in the park was published in 2012 (Delciellos et al. 2012). Since then some other rodent and bat species had their occurrence recorded for the first time in the SBNP and also in the state of Rio de Janeiro, such as *Drymoreomys albimaculatus* (Delciellos et al. 2015), *Phyllomys sulinus* (Delciellos et al. 2018a), and *Trinycteris nicefori* (Delciellos et al. 2018b). Regarding *Juliomys*, only a single specimen was previously captured in the park and firstly identified as *J. rimofrons* by external and dental morphology using diagnostic characteristics (Delciellos et al. 2012, Fonseca et al. 2013). The park encompasses areas of potential occurrence of all *Juliomys* species, except *J. ximenezi* (Christoff et al. 2016).

Field work was carried out at four sites in the municipality of Paraty, state of Rio de Janeiro, Brazil, along the state highway RJ-165 that crosses the SBNP (Figure 1). Sites range between 770 and 1,200 m a.s.l. (Table 1). The climate in the municipality of Paraty has a rainy season between October and April, and a drier season between May and September, with a mean annual precipitation of 2,284 mm and a mean annual temperature of 23.3ºC (Climate-Data.Org 2019). The vegetation is classified as dense montane rainforest (Delciellos et al. 2018a,b).

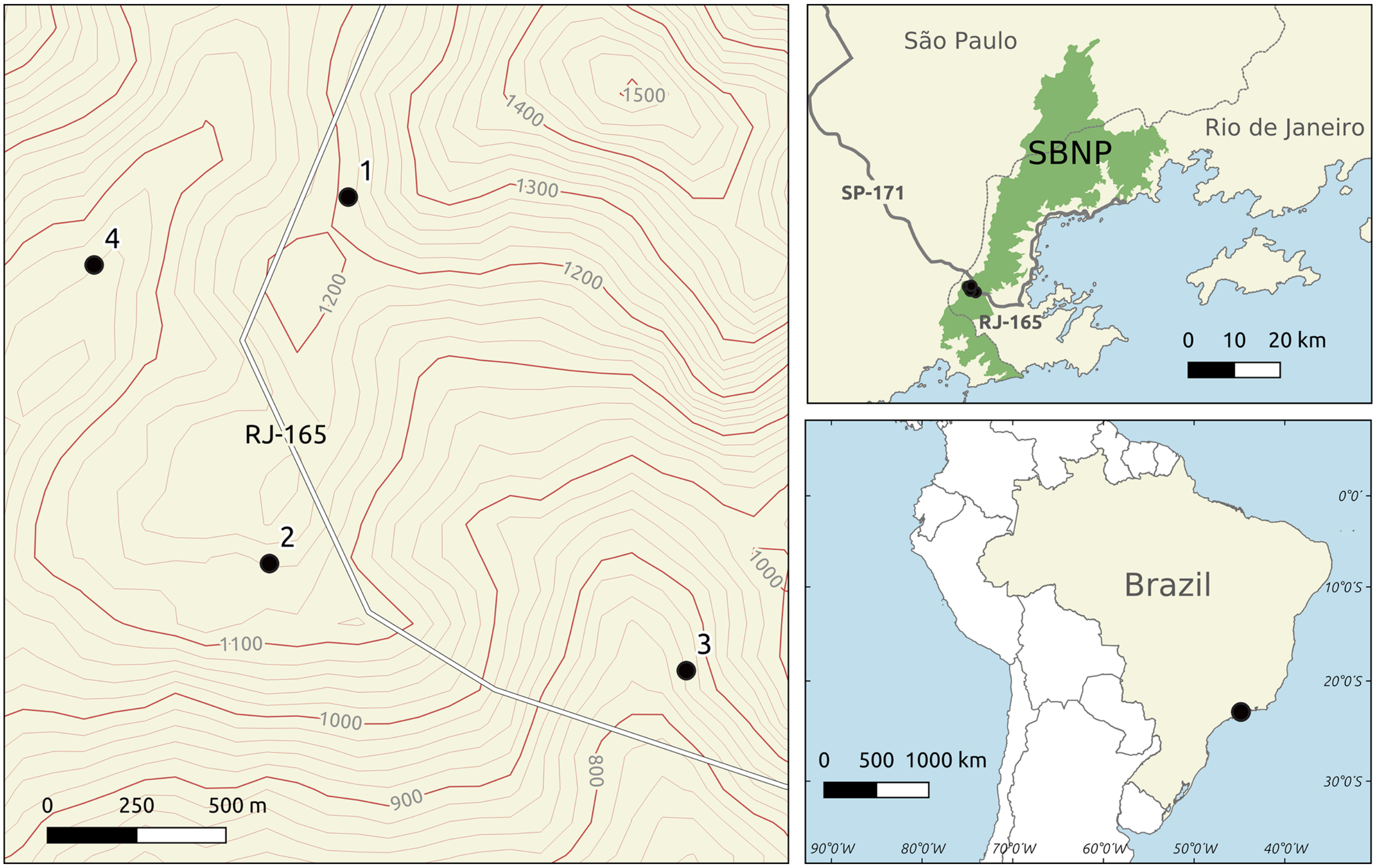

Figure 1. Collection localities of *Juliomys* (Rodentia: Sigmodontinae) specimens at the Serra da Bocaina National Park (SBNP). Localities numbers correspond to sites listed in Table 1.

Table 1. List of *Juliomys* (Rodentia: Sigmodontinae) specimens from which karyotype and/or cytochrome b sequence data were obtained from the Serra da Bocaina National Park, municipality of Paraty, state of Rio de Janeiro, Brazil. MN = Collected specimens deposited at the Museu Nacional, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil; BOCA = Field number from not collected specimens in the present study.

Taxon	Specimen number	Analysis	Site	Latitude	Longitude	Altitudinal range (m)
Juliomys ossitenuis	BOCA-1324	Cytochrome b	1	-23.189264	-44.837767	~1,170 – 1,200
Juliomys ossitenuis	MN 81807	Karyotype / Cytochrome b				
Juliomys ossitenuis	MN 81847	Karyotype / Cytochrome b				
Juliomys pictipes	BOCA-1233	Cytochrome b				
Juliomys ossitenuis	BOCA-1353	Cytochrome b	2	-23.198486	-44.839922	~1,100 – 1,125
Juliomys pictipes	BOCA-820	Cytochrome b	3	-23.201181	-44.828514	~770 – 810
Juliomys pictipes	BOCA-1231	Cytochrome b				
Juliomys pictipes	MN 81005	Cytochrome b				
Juliomys pictipes	MN 81843	Karyotype / Cytochrome b				
Juliomys pictipes	MN 83217	Cytochrome b				
Juliomys pictipes	MN 80998	Cytochrome b				
Juliomys ossitenuis	MN 81852	Karyotype / Cytochrome b	4	-23.190972	-44.844722	~1,050 – 1,100
Juliomys pictipes	BOCA-798	Cytochrome b				
Juliomys pictipes	BOCA-818	Cytochrome b				
Juliomys pictipes	BOCA-1157	Cytochrome b				
Juliomys pictipes	BOCA-1355	Cytochrome b				
Juliomys pictipes	MN 81841	Karyotype / Cytochrome b				
Juliomys pictipes	MN 83190	Karyotype				

2. *Sampling of small mammals*

Non-volant small mammals were sampled in twelve trap sessions from June 2013 to December 2016 with live and pitfall traps (IBAMA/MMA process no. 02001.003937/2008-18, authorization n. 248/2013 and 610/2015). At each site, two 290 m long transects were established, each with 30 trap stations, 10 m apart. At each transect, odd trap stations (N = 15) had a Tomahawk® placed on the floor; and even trap stations (N = 15) and the first 10 odd trap stations (N= 10) had a Sherman® in the understory between 1.5 and 2.5 m above ground. Tomahawk traps were baited with a mixture of banana, bacon, grinded peanut and oat, and Sherman traps with slices of banana. A sampling effort of 18,997 traps-night was carried out for livetraps.

Pitfall-traps, with 60 liters plastic buckets placed 10 m apart were arranged in transects with three to ten buckets, according to terrain characteristics, such as inclination and amount of rocks. Buckets were connected by a plastic-sheet drift fence 0.5 m high, buried 0.1 m below and extended perpendicularly to the ground, to induce the capture of wandering specimens. Each of the four sites had twenty buckets. For pitfalls, the sampling effort was 4,591 buckets-night.

Live and pitfall-traps remained active for five consecutive nights in each trap session. Trapped specimens were weighed using spring scales, sexed, measured (heady-body and tail lengths), and marked with a numbered ear-tag at the first capture (Ear tags, National Band & Tag Co., Newport, Kentucky, USA). In the field, specimens were identified as belonging to the genus *Juliomys* according to a set of external morphological characteristics, such as small size among sigmodontines, tail longer than the body, long pelage, orangish top of the head and on the rump, toes covered with whitish hairs, and short rostrum, as described by Bonvicino et al. (2008) and González et al. (2015). Collected specimens were prepared as vouchers and deposited at the Museu Nacional, Universidade Federal do Rio de Janeiro, Brazil. Specimens were handled following protocols approved by the American Society of Mammalogists (Sikes & Animal Care and Use Committee of the American Society of Mammalogists 2016).

3. *Karyotypic and molecular analysis*

Chromosomes in metaphases from six specimens (Table 1) were obtained with *in vitro* culture following Geise (2014) with modifications - culture kept at 36.5°C for 1 h 40 min. Slides with the karyotype preparation, stained with Giemsa 5%, were used to observe diploid (2n), fundamental autosomal numbers (FN), and chromosome morphology. Microscopic analyzes were done on the optic photomicroscope (Nikon Eclipse 50i), increase of 1,000 – immersion objective of 100 plus 10 ocular lenses. Karyotypes were assembled in descending order of chromosome size, two-arm chromosomes first, followed by acrocentric chromosomes, and compared with the literature. Karyotypes preparation and slides were deposited in the collection of the Laboratório de Mastozoologia, Universidade do Estado do Rio de Janeiro, Brazil.

Mitochondrial DNA of 17 *Juliomys* specimens was sequenced to help the correct identification of specimens (Table 1). Additionally, the *J. rimofrons* specimen (MN 77793) previously recorded in the study area (Delciellos et al. 2012, Fonseca et al. 2013) were also included in the analyses. Total genomic DNA was extracted from liver and epithelial tissue using the salt protocol and proteinase K (Bruford et al. 1992). The polymerase chain reaction (PCR) was performed using primers MVZ05 (5'-CGAAGCTTGATATGAAAAACCATCGTTG-3') and MVZ16 (5'-AAATAGGAARTATCAYTCTGGTTTRAT-3') (Smith & Patton 1993) for amplification of the first 801 pairs base (bp) of the mitochondrial cytochrome b gene (Cytb). For each PCR with a final volume of 10.0 µl were added 6.8 µL of ddH$_2$O, 1.0 µL of buffer 10× , 0.5 µL of MgCl$_2$ (50 mM), 0.2 µL of dNTP solution (10 mM each nucleotide), 0.2 µL of each forward and reverse primer (10 µM), 0.1 µL of Taq Platinum (Invitrogen Corporation) (5 U/µL) and 1.0 µL of DNA (40 ng/µL). For amplification the samples were exposed to the following conditions: initial denaturation at 94°C for 5 min, 39 cycles of annealing at 94°C for 30 sec, extension at 48°C for 45 sec and extension at 72°C for 45 sec, followed by a final extension step at 72°C for 5 min.

PCR products were purified with ExoSAP enzyme (GE Healthcare Life Sciences) and the sequencing reaction was performed with Big Dye v3.1 kit (Applied Biosystems) following the manufacturer's protocol. Subsequently, samples were sequenced in one direction only, using forward primer MVZ05 at ABI 3500 automated sequencer. Sequences were manually edited and aligned using ClustalW algorithm implemented in Molecular Evolutionary Genetics Analysis software (MEGA 7) (Kumar et al. 2017). We also included in the alignment 18 sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) (Table 2). We performed phylogenetic analyses using Maximum Likelihood (ML) with GTR + G (General Time Reversible + Gamma) substitution model. Nodal supports were evaluated using 100 bootstrap pseudoreplicates. Phylogenetic analyses were generated using RAxML v 8.2.9 software (Stamatakis 2014) at CIPRES platform (http://www.phylo.org/index.php). Only groups that presented bootstrap support equal to or above 70% were considered to be robust. *Rhagomys rufescens* and *Wiedomys cerradensis* sequences, taken from GenBank (http://www.ncbi.nlm.nih.gov/genbank/), were used as external groups (Table 2). The phylogeny obtained was edited manually using Figtree v1 4.3 (Rambaut 2016) and Inkscape 0.92.1 (https://inkscape.org/en/).

Results

Thirty-five species of non-volant small mammals were recorded from a total of 1,222 captured specimens. Twenty-four from these 1,222 specimens (2%) belong to the genus *Juliomys*, and six from these 24 specimens were collected, karyotyped and prepared as voucher specimens: three females (MN 81841, MN 81843, and MN 83190) were identified as *J. pictipes*, and two males and one female (MN 81807, MN 81847, and MN 81852) as *J. ossitenuis* (Table 1).

Table 2. List of Sigmodontinae specimens from which cytochrome b sequence data was used for phylogenetic analyses. * Holotype; ** Paratype. BOCA = Field number from not collected specimens in the present study. Zoological collections: AB = Museu de Zoologia, Universidade de São Paulo, São Paulo, Brazil (uncatalogued); MBML = Museu de Biologia Mello Leitão, Santa Teresa, Brazil; MCNU = Museu de Ciências Naturais, Universidade Luterana do Brazil, Canoas, Brazil; MLP = Museo de La Plata, Buenos Aires, Argentina; MN = Museu Nacional, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil; MZUSP = Museu de Zoologia, Universidade de São Paulo, São Paulo, Brazil; TK = Museum of Texas Tech University, USA; UFMG = Universidade Federal de Minas Gerais, Belo Horizonte, Brazil; UFPB = Universidade Federal da Paraíba, Brazil.

Taxon	Specimen number	GenBank number	Country: State	Source
Juliomys ossitenuis	AB 187	EF127521	Not provided by authors	Costa et al. (2007)
Juliomys ossitenuis	AB 196	EF127519	Brazil: São Paulo	Costa et al. (2007)
Juliomys ossitenuis	AB 258	EF127520	Brazil: São Paulo	Costa et al. (2007)
Juliomys ossitenuis	BOCA-1324	MT417218	Brazil: Rio de Janeiro	Present study
Juliomys ossitenuis	BOCA-1353	MT417217	Brazil: Rio de Janeiro	Present study
Juliomys ossitenuis	MBML 2783	EF127522	Brazil: Espírito Santo	Costa et al. (2007)
Juliomys ossitenuis	MN 81807	MT417219	Brazil: Rio de Janeiro	Present study
Juliomys ossitenuis	MN 81847	MT417216	Brazil: Rio de Janeiro	Present study
Juliomys ossitenuis	MN 81852	MT417215	Brazil: Rio de Janeiro	Present study
Juliomys ossitenuis	MN 69752*	EF127517	Brazil: Minas Gerais	Costa et al. (2007)
Juliomys ossitenuis	MZUSP 33170	EF127518	Brazil: São Paulo	Costa et al. (2007)
Juliomys ossitenuis	UFMG 3173**	EF127516	Brazil: Minas Gerais	Costa et al. (2007)
Juliomys pictipes	MN 77793	MT417228	Brazil: Rio de Janeiro	Present study
Juliomys pictipes	BOCA-1157	MT417232	Brazil: Rio de Janeiro	Present study
Juliomys pictipes	BOCA-1231	MT417225	Brazil: Rio de Janeiro	Present study
Juliomys pictipes	BOCA-1233	MT417224	Brazil: Rio de Janeiro	Present study
Juliomys pictipes	BOCA-1355	MT417229	Brazil: Rio de Janeiro	Present study
Juliomys pictipes	BOCA-798	MT417230	Brazil: Rio de Janeiro	Present study
Juliomys pictipes	BOCA-818	MT417226	Brazil: Rio de Janeiro	Present study
Juliomys pictipes	BOCA-820	MT417223	Brazil: Rio de Janeiro	Present study
Juliomys pictipes	MLP 1. I.03.24	EU157764	Argentina: Misiones	Pardiñas et al. (2008)
Juliomys pictipes	MN 81005	MT417221	Brazil: Rio de Janeiro	Present study
Juliomys pictipes	MN 81841	MT417227	Brazil: Rio de Janeiro	Present study
Juliomys pictipes	MN 81843	MT417222	Brazil: Rio de Janeiro	Present study
Juliomys pictipes	MN 83217	MT417231	Brazil: Rio de Janeiro	Present study
Juliomys pictipes	MN 80998	MT417220	Brazil: Rio de Janeiro	Present study
Juliomys pictipes	TK145073	FJ026733	Paraguay: Alto Parana	de la Sancha et al. (2009)
Juliomys pictipes	UFMG 3160	EF127515	Brazil: Minas Gerais	Costa et al. (2007)
Juliomys pictipes	UFMG 3168	EF127513	Brazil: São Paulo	Costa et al. (2007)
Juliomys pictipes	UFMG 3171	EF127514	Brazil: São Paulo	Costa et al. (2007)
Juliomys rimofrons	MN 46703**	AY029476	Brazil: Minas Gerais	Oliveira & Bonvicino (2002)
Juliomys rimofrons	MN 61647*	AY029477	Brazil: Minas Gerais	Oliveira & Bonvicino (2002)
Juliomys ximenezi	MCNU 464**	KT749862	Brazil: Rio Grande do Sul	Christoff et al. (2016)
Juliomys ximenezi	MCNU 868*	KT749863	Brazil: Rio Grande do Sul	Christoff et al. (2016)
Juliomys ximenezi	MCNU 869**	KT749864	Brazil: Rio Grande do Sul	Christoff et al. (2016)
Rhagomys rufescens	MN 66056	AY206770	Brazil: Minas Gerais	Percequillo et al. (2004)
Wiedomys cerradensis	UFPB9027	KM205445	Brazil: Maranhão	Olimpio et al. (2016)

For *J. pictipes*, the karyotype had a 2n of 36 and a FN of 34, and for *J. ossitenuis* 2n of 20 and FN of 36 (Figure 2). The autosomal complement of *J. pictipes* was composed only by acrocentric chromosomes in decreasing size and the X chromosome was a medium acrocentric. The karyotype of *J. ossitenuis* was composed of six metacentric and three submetacentric chromosome pairs. The X chromosome was a submetacentric and the Y an acrocentric. In the Cytb phylogeny, 12 specimens grouped with *J. pictipes*, and five with *J. ossitenuis* (Table 2; Figure 3). The specimen previously identified as *J. rimofrons* (MN 77793) clustered together with *J. pictipes* (Figure 3).

Two *Juliomys* specimens were captured with Sherman traps placed in the understory, and 91.7% (N = 22) of captures occurred on pitfalls. Specimens of *J. pictipes* were captured in sites 1, 3 and 4, in altitudes ranging from 770 to 1,200 m a.s.l. (Table 3). For this species, the highest number of captures occurred in sites 3 and 4, with six captures each. Specimens of *J. ossitenuis* were captured in sites 1, 2, and 4, in altitude ranging from 1,050 to 1,200 m a.s.l. (Table 3). For this species, most specimens (3 out of 5) were captured in site 1, which is the site with the highest altitude. Sympatry between these two *Juliomys* species occurred on sites 1 and 4 (Table 3). Also, in trap sessions 7 (January 2015) and 10 (February 2016) both species were captured, but not at the same site (Table 3).

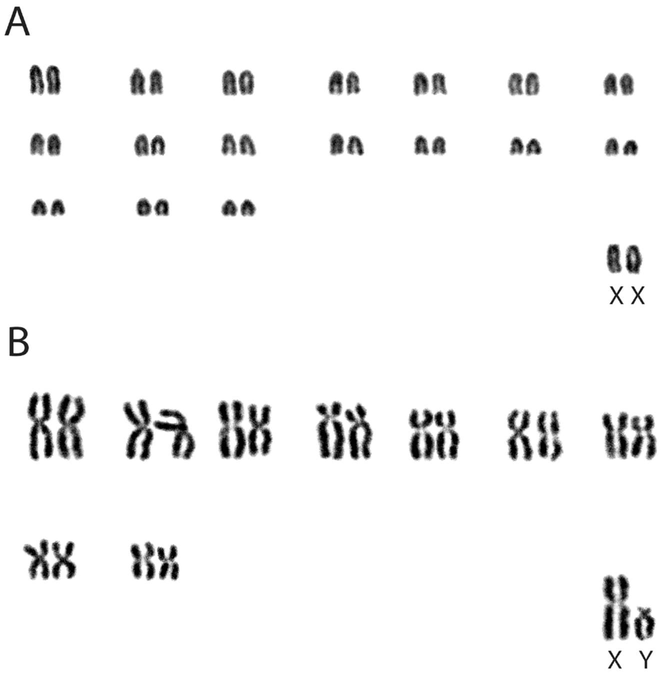

Figure 2. Conventional staining of the karyotypes of *Juliomys pictipes* (A – MN 83190, female) and *Juliomys ossitenuis* (B – MN 81807, male), showing 2n = 36, FN =34 and 2n = 20, FN = 36, respectively.

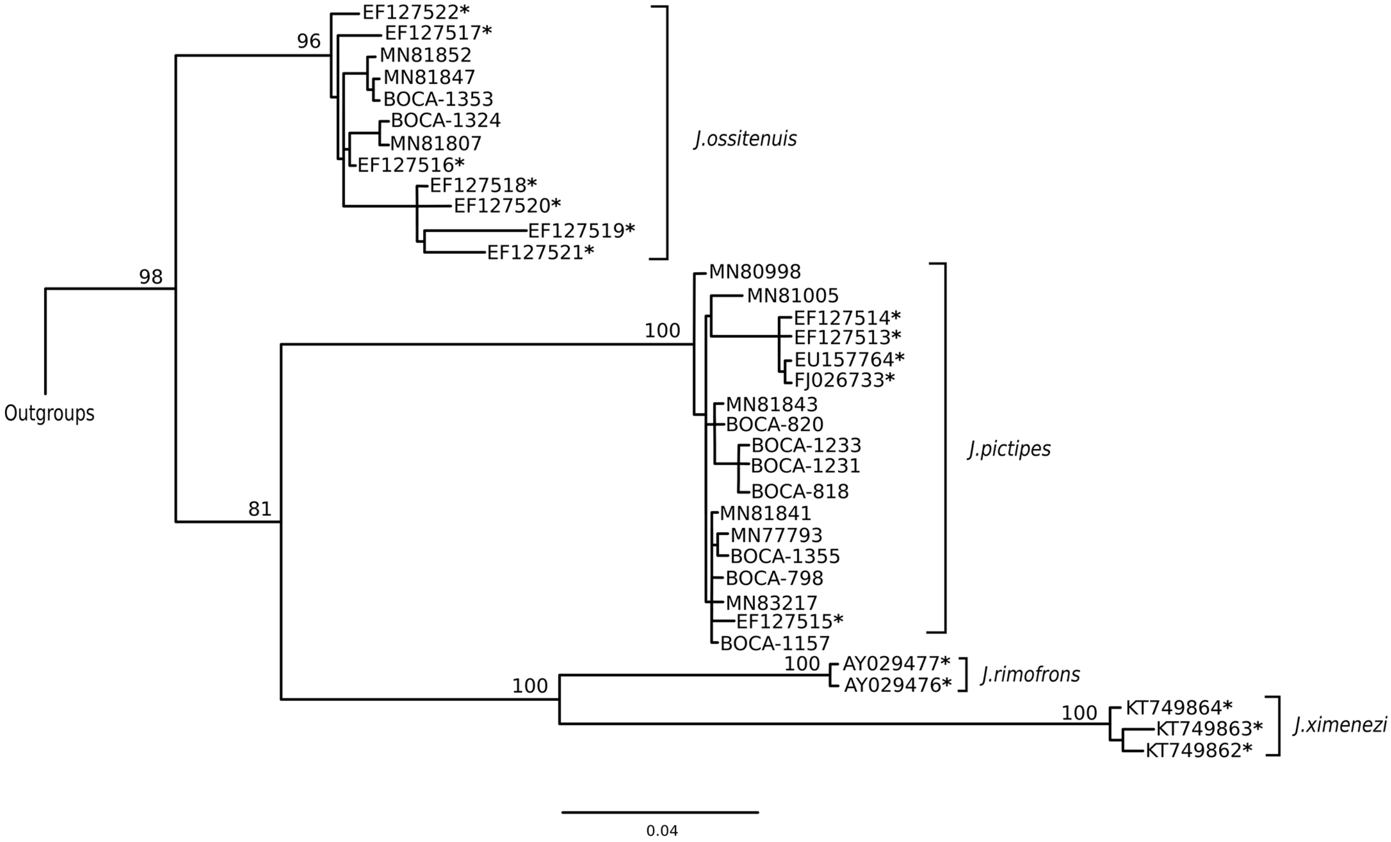

Figure 3. Phylogenetic relationships among *Juliomys* (Rodentia: Sigmodontinae) species based on maximum likelihood using 801 base pairs of cytochrome b (Cytb) gene under GTR + G model of sequence evolution. Bootstrap support values (> 70%) from maximum likelihood are indicated in the nodes. Specimens from GenBank are followed by asterisk. Scale bar corresponds to the mean number of nucleotide substitutions per site.

Table 3. Occurrence of *Juliomys pictipes* (JP) and *Juliomys ossitenuis* (JO) (Rodentia: Sigmodontinae) during trap sessions carried out from June 2013 to December 2016 along an altitudinal gradient at the Serra da Bocaina National Park, municipality of Paraty, state of Rio de Janeiro, Brazil. Sympatry between these two species occurred on sites 1 and 4. In trap sessions 7 and 10 both species were captured, but not at the same site. Trap sessions: 1 = June 2013; 2 = September 2013; 3 = December 2013; 4 = April 2014; 5 = June 2014; 6 = October 2014; 7 = January 2015; 8 = October 2015; 9 = December 2015; 10 = February 2016; 11 = July 2016; 12 = December 2016.

Site	Altitudinal range (m a.s.l.)	Trap sessions											
		1	2	3	4	5	6	7	8	9	10	11	12
1	~1,170 – 1,200				JO			JO		JP	JO		
2	~1,100 – 1,125										JO		
4	~1,050 – 1,100				JO		JP	JP	JP		JP		
3	~770 – 810	JP						JP	JP	JP			

Discussion

The karyological and molecular analyses carried out provided the detection of two *Juliomys* species in the SBNP for the first time: *J. pictipes* and *J. ossitenuis*. On the other hand, the previous record of *J. rimofrons* in the park (site 3) based on the specimen MN 77793 (Delciellos et al. 2012, Fonseca et al. 2013) should be disregarded, as this specimen is actually a *J. pictipes* and not *J. rimofrons* according to our molecular analyses results. Thus, we confirmed the occurrence of only two *Juliomys* species in the park. Additionally, *J. pictipes* and *J. ossitenuis* co-occurred in two out of four sites with altitudes higher than 1,000 m a.s.l., representing the fifth known record of sympatry between these species up to date.

The first studies carried out at the SBNP evidenced the occurrence of *J. rimofrons* in the park (Delciellos et al. 2012, Fonseca et al. 2013). Fonseca et al. (2013) used solely external and dental morphology for species identification, which were based mainly on the presence of the interfrontal fontanelle and small ectolophid and ectostylid of lower molars m1 and m2. These are well-established characters that can be used to differentiate *J. rimofrons* from the other three *Juliomys* species (Costa et al. 2007, Christoff et al. 2016). However, in the present study the specimen previously identified as *J. rimofrons* (MN 77793) by Fonseca et al. (2013) clustered with *J. pictipes* in the phylogeny based on cytochrome b sequences. Thus, the occurrence of *J. rimofrons* in the Serra da Bocaina remains to be confirmed in future studies, as well as the species identification of the second *J. rimofrons* specimen (MN 76236) analyzed by Fonseca et al. (2013), collected at the municipality of São José do Barreiro, State of São Paulo, has not yet been confirmed through molecular analyzes. The phylogeny based on cytochrome b sequences also allowed the first record of *J. pictipes* and *J. ossitenuis* in the park. Karyotypes found by us for *J. pictipes* (Bonvincino & Otazu 1999, Aguieiras et al. 2013, Di-Nizo et al. 2014) and *J. ossitenuis* (Costa et al. 2007, Paresque et al. 2009, Aguieiras et al. 2013) are in accordance with the literature.

Since Poulton (1904) coined the term "sympatry", its meaning has been extensively debated by ecologists (see discussion in Mallet et al. 2009). Here sympatry was defined as the co-occurrence of species in the same locality, in which individuals of both species may encounter one another with frequency but there may be an ecological segregation between them (i.e., species have different niche requirements) (Mallet et al. 2009). Sympatry between *J. pictipes* and *J. ossitenuis* in the SBNP was detected in sites above 1,050 m a.s.l. where both species were expected to occur according to our results and those of previous studies (Costa et al. 2007, Pardiñas et al. 2008, Cerboncini et al. 2014).

Juliomys ossitenuis is an endemic species of high altitudes (> 800 m) in the Brazilian Atlantic Forest, while *J. pictipes* is endemic to the Atlantic Forest but with a larger extent of occurrence than *J. ossitenuis*, inhabiting altitudes that range from sea level to 2,000 m a.s.l. (Geise & Pardiñas 2016). Thus, the altitudinal gradient observed in the Atlantic Forest may play a role in establishing sympatry areas between these species, because these areas are expected to occur only between 800 and 2,000 m a.s.l.. Several extrinsic factors vary along an altitudinal gradient, such as vegetation characteristics, temperature, precipitation, topography, and soil characteristics, among others (Graham et al. 2014). This complex environmental heterogeneity found in an altitudinal gradient can affect from individual's tolerance to abiotic factors (e.g., temperature) to community patterns of co-occurrence (e.g., species richness), being these spatial patterns of biodiversity extensively studied in community ecology (Graham et al. 2014, Willig & Presley 2016). The specific factors that cause *J. ossitenuis* to occur only at high altitudes in the Atlantic Forest also remains to be evaluated in future studies.

Despite the recognized role of mountain systems to promote isolation and species diversification (Graham et al. 2014), *J. pictipes* and *J. ossitenuis* are not sister species (see Figure 3) and therefore these records of sympatry in the SBNP may represent a secondary contact zone. Pardiñas et al. (2008) also suggested a relatively old separation between these two species. The explanatory factors for the occurrence of sympatry between these species in the park were not evaluated in the present study because of the small data set available, but sympatry among similar or cryptic species frequently is possible due to habitat and diet partitioning (Mallet et al. 2009). *Juliomys pictipes* and *J. ossitenuis* specimens did not differ in forest strata where captures occurred in the present study, but these species may have different habitat preferences or behaviors related to the use of three-dimensional space that have not yet been clarified. Additionally, *J. pictipes* and *J. ossitenuis* seem not differ greatly in diet, with both species considered granivorous according to Galetti et al. (2015) and González et al. (2015).

Juliomys pictipes and *J. ossitenuis* can be considered as rare species in the communities where they occur (e.g., Pardini et al. 2005).

This rarity in local communities was corroborated in the present study, as *Juliomys* specimens accounted for only 2% of captured specimens. This rarity of *Juliomys* species in small mammals' communities, associated with species misidentifications in the field using solely external morphological characteristics, and infrequent use of pitfalls traps by researchers, may be contributing with the few existing records of sympatry between these species.

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Author Contributions

Ana Cláudia Delciellos: Substantial contribution in the concept and design of the study; contribution to data collection; contribution to data analysis and interpretation; contribution to manuscript preparation; contribution to critical revision, adding intellectual content.

Marcia Aguieiras: Contribution to data collection; contribution to data analysis and interpretation; contribution to manuscript preparation; contribution to critical revision, adding intellectual content.

Gabriela Colombo de Mendonça: Contribution to data collection; contribution to data analysis and interpretation; contribution to manuscript preparation; contribution to critical revision, adding intellectual content.

Ana Carolina Loss: Contribution to data collection; contribution to data analysis and interpretation; contribution to manuscript preparation; contribution to critical revision, adding intellectual content.

Oscar Rocha-Barbosa: Substantial contribution in the concept and design of the study.

Lena Geise: Contribution to data collection; contribution to data analysis and interpretation; contribution to manuscript preparation; contribution to critical revision, adding intellectual content.

Conflicts of Interest

The authors declare that they have no conflict of interest related to the publication of this manuscript.

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Inventory

Ichthyofauna of sandy beaches along the Acre river, Brazil

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Abstract: Despite increasing efforts in recent years to catalog the fish diversity of Amazonian rivers, many regions are still under-sampled, and sandy beach environments are particularly poorly understood. The present study focused on a 300 km stretch of the Acre river, in the southwestern Amazon basin, where we sampled 30 sandy beaches separated by a mean interval of 10 km. We collected 15,329 fish representing 80 species, 26 families, and nine orders. The Characiformes were the most abundant order, providing 88.24% of the individuals collected, followed by the Siluriformes, with 10.03%, while the Siluriformes had the highest species richness, with 37 species (45.0%), followed by the Characiformes, with 30 (37.5%). The most abundant species were the characiforms *Knodus orteguasae* and *Creagruto barrigai*. Reliable data on a region's biota is fundamental for the evaluation of patterns of biodiversity, and the occurrence and management of threatened species. As fish are directly affected by pollutants and the degradation of aquatic environments, further research in areas that are still poorly sampled will be essential for the elaboration of effective conservation strategies.

Keywords: *Amazon; Characiformes; Neotropical Region; Sampling Gaps; Siluriformes; Species Diversity.*

Ictiofauna de praias arenosas ao longo do rio Acre, Brasil

Resumo: Apesar dos crescentes esforços para catalogar a diversidade de peixes nos rios amazônicos, muitas regiões ainda estão sub-amostradas e os ambientes de praias arenosas são particularmente pouco compreendidos. Este estudo foi realizado ao longo de um trecho de 300 km do Rio Acre, no sudoeste da bacia amazônica, onde foram amostradas 30 praias, separadas por uma distância média de 10 km. Foram coletados 15.329 peixes, representados em 80 espécies, 26 famílias e nove ordens. Characiformes foi a ordem mais abundante, representando 88,24% dos indivíduos coletados, seguidos pelos Siluriformes, com 10,03%, enquanto os Siluriformes apresentaram a maior riqueza, com 37 espécies (45,0%), seguidas pelos Characiformes, com 30 espécies (37,5%). As espécies mais abundantes foram os characiformes *Knodus orteguasae* e *Creagruto barrigai*. Dados confiáveis sobre a biota de uma região são fundamentais para a avaliação dos padrões de biodiversidade e conhecimento sobre a ocorrência e manejo de espécies ameaçadas. Como os peixes são diretamente afetados por poluentes e pela degradação dos ambientes aquáticos, mais pesquisas em áreas que ainda são pouco amostradas serão essenciais para a elaboração de estratégias eficazes de conservação.

Palavras-chave: *Amazônia; Characiformes; Diversidade de espécies; Lacunas de amostragem; Região Neotropical; Siluriformes.*

Introduction

The hydrographic network of the Neotropical region supports the world's most diverse freshwater fish fauna, with approximately 5,160 species (Reis et al. 2016, Jézéquel et al. 2020). This diversity is likely still underestimated, and recent predictions have pointed to a final total of between 8000 and 9000 species (Albert & Reis 2011, Reis et al. 2016). The basin of the Neotropical Amazon river is the world's largest and most diverse freshwater system, with a total area of approximately 7 million km^2, representing 20% of all the freshwater discharged into the oceans (Callède et al. 2010). Up to now, 2406 fish species have been recorded in the Amazon basin, including approximately 1402 endemic forms, distributed in 514 genera, 56 families, and 18 taxonomic orders (Dagosta & de Pinna 2019, Jézéquel et al. 2020). This fauna is distributed throughout an ample diversity of aquatic systems, including major rivers, lakes, streams, floating vegetation, and beaches (Beltrão et al. 2019, Oberdorff et al. 2019). Beaches are key environments for the maintenance of regional fish diversity, in particular due to their provision of shelter for many species (Olds et al. 2018).

Fluvial beaches are areas formed mainly by deposits of sand and clay carried by rivers, primarily during the flood period, and are present on approximately half of the area of river margin in the Amazon basin. The fish fauna found in these environments is very diverse (Goulding 1997, Lowe-McConnell 1999, Py-Daniel et al. 2007, Duarte et al. 2010), composed mainly of small species with diverse feeding habits and reproductive strategies. This enormous diversity of species is linked to several evolutionary factors, including the formation of the drainage basins, hydrological dynamics, environmental heterogeneity, and flood and reflux pulses (Val 2019).

A number of studies have recorded greater fish species richness in beach environments on rivers of the Amazon basin, in comparison with other aquatic systems, such as lakes, streams, and floating vegetation (Py-Daniel et al. 2007). In an early ichthyological survey of the Negro river, Lowe-McConnell (1989) recorded 488 species, of which 248 were found in beach environments, 184 in flooded forests, and 56 under floating aquatic macrophytes. On the Madeira river, Py-Daniel et al. (2007) compiled a list of 247 species, with 119 in beach environments, 32 in the main channel, 44 under floating macrophytes, and 52 in streams. The high diversity of fish recorded in beach environments may be related to the nutrient dynamics and availability of habitats in these environments, which have a direct influence on energy flow and favor species richness (Lowe-McConnell 1999, Roach & Winemiller 2015). On the lower Purus river, in the westernmost Amazon basin, Duarte et al. (2010) studied the ichthyofauna of beach environments, while Py-Daniel & Deus (2003) surveyed the local ichthyofauna and commented on local fisheries. Silva et al. (2010) studied the structure and dynamics of the fish communities of streams in the flood zone, while Morales et al. (2019) surveyed the ichthyofauna of the floodplain lakes of the Piagaçu-Purus Sustainable Development Reserve, also on the Purus river.

The Acre river is a medium-sized watercourse, by the standards of the Amazon basin, running 1190 km from its source, in Peru, to its confluence with the Purus river in the Brazilian state of Amazonas, first passing through Bolivia and then the state of Acre, Brazil. Ichthyological research in Acre has included studies on the Juruá river (Silvano et al. 2001) and the middle Purus (Dos Anjos et al. 2008), as well as studies of the streams (Claro-García et al. 2013, Corrêa et al. 2018, Virgilio et al. 2018, 2019) and lakes (da Silva et al. 2013) of the Acre basin. Other specific studies on the Acre river have included the evaluation of the conservation status of the river's fish in Bolivia (Añez et al. 2010) and the analysis of the population growth patterns of some fish species that inhabit beach environments in the Brazilian stretch.

Although the number of studies in the region has increased over time, considerable sampling gaps persist, and beach environments are particularly under-sampled. Data on the diversity, ecology, and distribution of fishes are essential for the development of effective conservation strategies for both threatened species and areas that have been affected by anthropogenic impacts (Closs et al. 2016). To help revert this scenario, the present study provides a comprehensive checklist of the ichthyofauna of sandy beaches along the Acre river. We also used the data to obtain an estimate of the of fish species richness of these beach environments.

Material and methods

1. *Study area*

The present study focused on an area between the municipalities of Brasiléia (11°1'1.56" S, 68°44'38.51" W) and Rio Branco (10°9'21.84" S, 67°49'4.86" W), in the state of Acre, Brazil. A total of 30 beaches were sampled over a 300 km stretch of the Acre river, with a mean interval of 10 km between each pair of sites (Figure 1). The majority of the Acre basin (27,263 Km2) is located within the Brazilian state of Acre, prior to flowing into the Purus river in the neighboring state of Amazonas, Brazil. The principal tributary of the Acre river is the Riozinho do Rola, while other important affluent include the Xapuri, Antimary, and Andirá rivers.

2. *Ichthyofauna sampling*

Two samplings were performed between August and September 2017, at the end of the region's dry season. At each site, we used a trawl net, 9 m long and 2 m in height, with a 5 mm mesh. We collected fish both during the day (between 6h and 10h) and at night (between 18h and 21h), with three parallel trawls being conducted during each session, in order to guarantee an adequate sample of the local fish assembly. Even so, some species were only acquired from third parties or by other collection techniques, i.e., by line fishing. These techniques were used selectively within the area of the beach, targeting either the deepest water or other environments that could not be trawled. Similarly, specimens donated by third parties were only included in the sample when they were known to have been collected within the area of the beach.

The morphometric measurements were taken on the left side of the body, using a digital caliper with an accuracy of 1 mm. The specimens were anesthetized in aqueous lidocaine solution, fixed in 10% formalin, and packed in plastic bags. The species were identified at the Ichthyology and Aquatic Ecology Laboratory of the Universidade Federal do Acre (UFAC), based on taxonomic keys and works (e.g., Albert et al. 2012, Queiroz et al. 2013, Brito et al. 2018) and, when necessary, specialists were consulted. The taxonomic nomenclature followed Fricke et al. (2020). After identification, the fish were transferred to 70% alcohol and some specimens were deposited at the UFAC fish collection in Rio Branco. The specimens were collected under permanent collection license no. 11185, emitted by the Brazilian Biodiversity Authorization and Information System (SISBio).

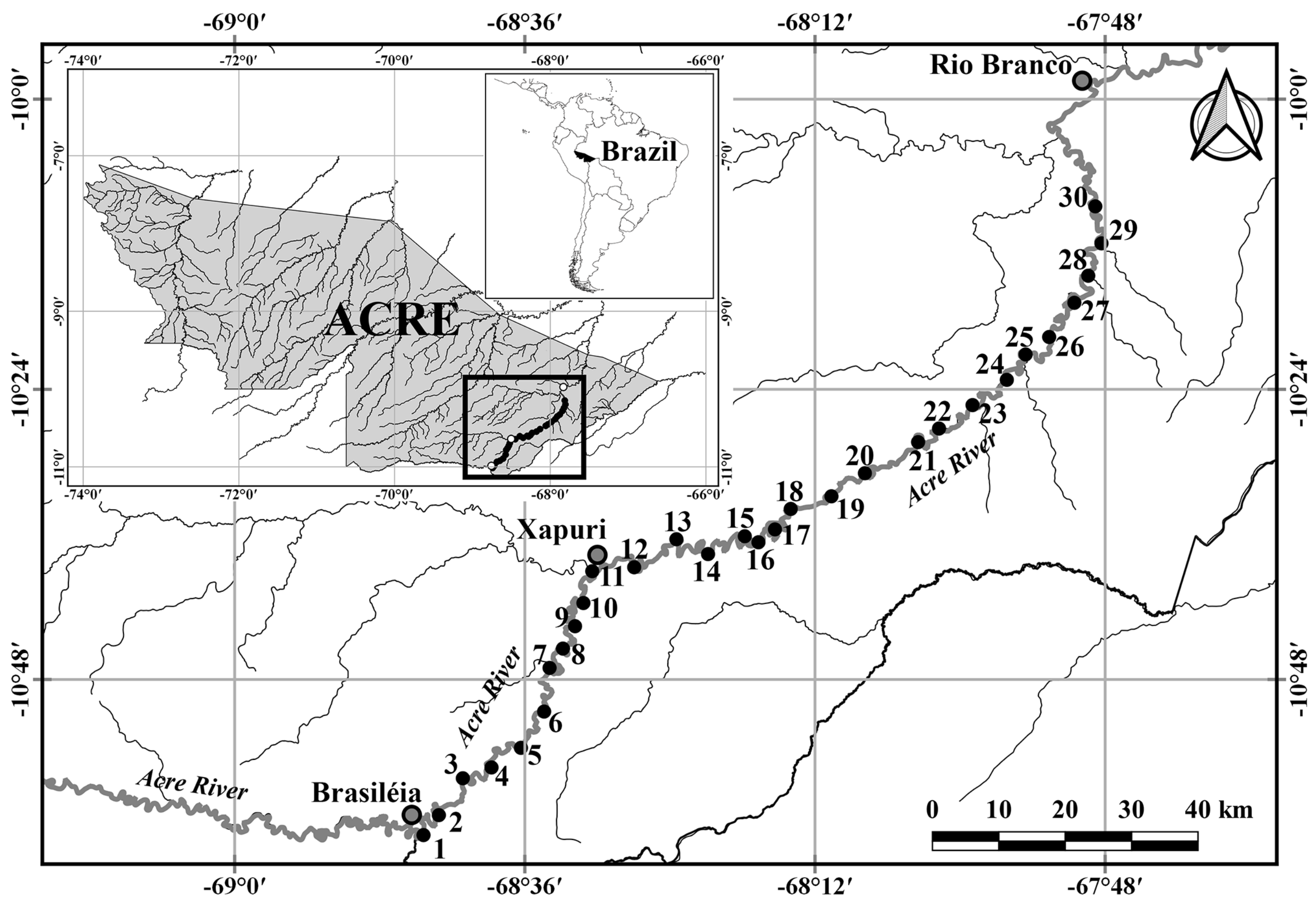

Figure 1. Beaches sampled along a 300 km stretch of the Acre river, between the municipalities of Brasiléia and Rio Branco in the state of Acre, Brazil.

3. *Statistical analysis*

The exploratory analyzes of the data considered all the species recorded during the study period. To estimate species richness, however, only the species caught in the trawls were included, given that this collection method was standardized at all sampling sites. Species richness was estimated using a sampled-based accumulation curve, with 999 permutations of the abundance matrix, with the rows corresponding to the sites and the columns to the species. We used the Jackknife 1 estimator to obtain the expected richness. This analysis was run in EstimateS® (Colwell & Elsensohn 2014).

Results

We collected a total of 15,329 individuals representing 80 species, 26 families, and nine orders (Table 1). The order Siluriformes had the highest species richness (37 species), followed by the Characiformes (30 species), and Gymnotiformes (4 species). The Characiformes was the most abundant order (88.58% of the individuals collected), followed by the Siluriformes (10.03%), and the Perciformes (1.08%) (Figure 2A).

The families with the highest species richness and abundance were the Characidae (14 species and 12,931 individuals), followed by the Loricariidae (12 species and 950 individuals), and the Pimelodidae, with 10 species and 364 individuals (Figure 2B). The most abundant species were *Knodus orteguasae* (Fowler, 1943), with 76.25% of the individuals, followed by *Aphanotorulus unicolor* (Steindachner, 1908), with 4.68%, *Creagrutus barrigai* Vari & Harold, 2001 (4.59%), *Engraulisoma taeniatum* Castro, 1981 (2.35%), and *Clupeacharax anchoveoides* Pearson, 1924, with 2.13% of the individuals (Figure 2C). The other 75 species represented 10% of the total, and 30 were considered rare, being represented by only a single individual. The beach with the most diverse fish fauna was S08 (25 species), followed by S21 (22 species), and S18 and S24, each with 20 species. The sites with the greatest abundance of fish were S19 (1391 individuals), S27 (1151), S11 (1079), and S18, with 1,069 individuals being collected (Figure 3). We obtained 60 fish species in the trawls and 20 by alternative methods (third parties).

The observed species richness (60 species) represented 77.92% of the richness estimated by Jackknife 1 (77±5 species). The accumulation curve presented a tendency to stabilize, but did not reach an asymptote, indicating that even more species would be recorded with increasing sampling effort (Figure 4). The fish species recorded in the present study are shown in Figures 5–9.

Discussion

The fish species richness recorded in the present study, in the sandy beach habitats of the Acre river is greater than that found in other aquatic environments within the same basin, such as lakes (53 species; da Silva et al. 2013) and streams (34 species; Ramalho et al. 2014), although

Table 1. Fish species recorded in sandy beach environments of the Acre river in the present study, indicating the capture period (CP; D = day; N = night; D/N = day and night), the mean total length (TL) of the specimens collected, the abundance (AB) of individuals, and the catalog numbers (CN) of the voucher specimens deposited in the UFAC fish collection in Rio Branco, Brazil. Species with no data on the capture period were donated by third parties. *Species classified as Critically Endangered in the Brazilian Red Book of Endangered Fauna. The orders and families are classified according to Fricke et al. (2020).

TAXA	SP	TL (mm)	AB	CN
MYLIOBATIFORMES				
Potamotrygonidae				
Paratrygon aiereba (Müller & Henle, 1841)*	–	445±55.1	3	MUFAC–IC1146
Potamotrygon cf. *orbignyi*	–	679.5±85.56	2	MUFAC–IC1147
CLUPEIFORMES				
Pristigasteridae				
Pellona castelnaeana (Valenciennes,1847)	–	–	–	MUFAC–IC1220
CHARACIFORMES				
Crenuchidae				
Characidium cf. *steindachneri*	D	43.6±3.6	17	MUFAC–IC1177
Curimatidae				
Psectrogaster amazonica Eigenmann & Eigenmann, 1889	–	128±10.9	9	MUFAC–IC1166
Psectrogaster rutiloides (Kner, 1858)	N	131	1	MUFAC–IC1167
Steindachnerina guentheri (Eigenmann & Eigenmann, 1889)	D	90±6.6	4	MUFAC–IC1168
Steindachnerina leucisca (Günther, 1868)	N	140	1	MUFAC–IC1169
Steindachnerina pupula Vari, 1991	D/N	101.4±17.3	41	MUFAC–IC1170
Prochilodontidae				
Prochilodus nigricans Spix & Agassiz, 1829	N	211	1	MUFAC–IC1176
Anostomidae				
Abramites hypselonotus (Günther, 1868)	D	178±123	2	MUFAC–IC1148
Serrasalmidae				
Serrasalmus maculatus Kner, 1858	–	138	1	MUFAC–IC1171
Mylossoma duriventre (Cuvier, 1818)	–	159	1	MUFAC–IC1172
Cynodontidae				
Rhaphiodon vulpinus Spix & Agassiz, 1829	–	282	1	MUFAC–IC1173
Gasteropelecidae				
Thoracocharax stellatus (Kner, 1858)	D/N	53.8±8.6	195	MUFAC–IC1175
Characidae				
Aphyocharax pusillus (Günther, 1868)	D/N	56.42±2.13	8	MUFAC–IC1149
Astyanax abramis (Jenyns, 1842)	D	75	1	MUFAC–IC1150
Astyanax bimaculatus (Linnaeus, 1758)	D/N	134.5±42.8	6	MUFAC–IC1151
Creagrutus barrigai Vari & Harold, 2001	D/N	31.3±7.4	703	MUFAC–IC1153
Ctenobrycon spilurus (Valenciennes, 1850)	D/N	61.8±8.3	8	MUFAC–IC1154
Galeocharax gulo (Cope, 1870)	D/N	73.8±39.4	28	MUFAC–IC1156
Knodus orteguasae (Fowler, 1943)	D/N	32±10.9	11690	MUFAC–IC1157
Leptagoniates steindachneri Boulenger, 1887	D/N	58.2±5	13	MUFAC–IC1158
Moenkausia sp. “lepidura alta”	D/N	57.7±5.3	38	MUFAC–IC1159
Odontostilbe fugitiva Cope, 1870	D/N	34.5±2.7	63	MUFAC–IC1160

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Paragoniates alburnus Steindachner, 1876	D/N	58.6±7.3	17	MUFAC–IC1161
Prionobrama filigera (Cope, 1870)	D/N	49.5±9	24	MUFAC–IC1162
Protocheirodon pi (Vari, 1978)	D/N	40.3±4.9	4	MUFAC–IC1163
Tetragonopterus argenteus Cuvier, 1816	D	84.5±2.1	2	MUFAC–IC1164
Triportheidae				
Clupeacharax anchoveoides Pearson, 1924	D/N	36.3±15.5	327	MUFAC–IC1152
Engraulisoma taeniatum Castro, 1981	D/N	28.3±4.4	360	MUFAC–IC1155
Triportheus albus Cope, 1872	D/N	132.9±11.9	13	MUFAC–IC1165
Bryconidae				
Salminus sp.	D	200	1	MUFAC–IC1174
GYMNOTIFORMES				
Rhamphichthyidae				
Gymnorhamphichthys hypostomus Ellis, 1912	N	255.5±23.3	2	MUFAC–IC1212
Sternopygidae				
Eigenmannia virescens (Valenciennes, 1836)	D/N	141.9±38.7	18	MUFAC–IC1213
Apteronotidae				
Sternarchogiton nattereri (Steindachner, 1868)	N	162	1	MUFAC–IC1210
Sternarchorhynchus chaoi de Santana & Vari, 2010	D/N	210±35.4	2	MUFAC–IC1211
SILURIFORMES				
Aspredinidae				
Amaralia hypsiura (Kner, 1855)	D	75	1	MUFAC–IC1178
Micromyzon cf. *akamai*	N	18	1	MUFAC–IC1219
Trichomycteridae				
Henonemus punctatus (Boulenger, 1887)	D/N	87.4±18	23	MUFAC–IC1207
Pseudostegophilus nemurus (Günther, 1869)	D/N	99.7±29.6	24	MUFAC–IC1208
Vandellia cirrhosa Valenciennes, 1846	D/N	62±8.5	27	MUFAC–IC1209
Loricariidae				
Ancistrus sp.	D	77	1	MUFAC–IC1188
Aphanotorulus unicolor (Steindachner, 1908)	D/N	39.61±26.5	717	MUFAC–IC1189
Farlowella nattereri Steindachner, 1910	D	95±5.7	3	MUFAC–IC1191
Hypostomus cf. *pyrineusi*	D	122±42.4	2	MUFAC–IC1192
Lamontichthys filamentosus (La Monte, 1935)	D	300.5±45.9	2	MUFAC–IC1193
Limatulichthys griseus (Eigenmann, 1909)	D/N	207.5±15.4	6	MUFAC–IC1194
***Loricaria* sp.**	–	233.9±154.2	11	MUFAC–IC1195
Panaqolus purusiensis (La Monte, 1935)	–	124	1	MUFAC–IC1196
Peckoltia brevis (La Monte, 1935)	–	111.7	1	MUFAC–IC1197
Rhadinoloricaria bahuaja (Chang & Castro, 1999)	D/N	103.9±107.7	202	MUFAC–IC1190
Spatuloricaria cf. *puganensis*	–	361	1	MUFAC–IC1198
Sturisoma lyra (Regan, 1904)	D	284	1	MUFAC–IC1199
Heptapteridae				
Imparfinis guttatus (Pearson, 1924)	D	95	1	MUFAC–IC1186
Pimelodella howesi Fowler, 1940	D/N	79.6±29.2	131	MUFAC–IC1187

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Doradidae				
Leptodoras acipenserinus (Günther, 1868)	–	260	1	MUFAC–IC1183
Nemadoras sp.	–	150	1	MUFAC–IC1184
Oxydoras niger (Valenciennes, 1821)	–	625	1	MUFAC–IC1185
Pterodoras granulosus (Valenciennes, 1821)	–	–	–	MUFAC–IC1225
Auchenipteridae				
Auchenipterus nuchalis (Spix & Agassiz, 1829)	N	185	1	MUFAC–IC1179
Centromochlus heckelii (De Filippi, 1853)	N	101.7±24.5	7	MUFAC–IC1180
Centromochlus perugiae Steindachner, 1882	N	28.3±3.5	3	MUFAC–IC1181
Tympanopleura piperata Eigenmann, 1912	N	103	1	MUFAC–IC1182
Pimelodidae				
Calophysus macropterus (Lichtenstein, 1819)	–	–	–	MUFAC–IC1221
Cheirocerus eques Eigenmann, 1917	D/N	78.8±56.1	144	MUFAC–IC1200
Exallodontus aguanai Lundberg, Mago-Leccia & Nass, 1991	–	–	–	MUFAC–IC1222
Leiarius marmoratus (Gill, 1870)	–	–	–	MUFAC–IC1223
Megalonema amaxanthum Lundberg & Dahdul, 2008	D/N	55±35.4	204	MUFAC–IC1201
Megalonema platycephalum Eigenmann, 1912	D/N	81.6±59.8	5	MUFAC–IC1202
Pimelodus blochii Valenciennes, 1840	N	209	1	MUFAC–IC1203
Pimelodus cf. *maculatus*	N	148.5±36.9	5	MUFAC–IC1204
Platysilurus mucosus (Vaillant, 1880)	–	148	1	MUFAC–IC1205
Sorubim lima (Bloch & Schneider, 1801)	N	290	1	MUFAC–IC1206
PLEURONECTIFORMES				
Achiridae				
Apionichthys finis (Eigenmann, 1912)	D/N	86.5±2.1	2	MUFAC–IC1215
CICHLIFORMES				
Cichlidae				
Bujurquina syspilus (Cope, 1872)	D	30.5±13.7	4	MUFAC–IC1217
Crenicichla sp. “Juvenil”	D/N	28±4.5	6	MUFAC–IC1218
BELONIFORMES				
Belonidae				
Pseudotylosurus angusticeps (Günther, 1866)	D/N	148.2±19.8	5	MUFAC–IC1214
PERCIFORMES				
Sciaenidae				
Pachypops pigmaeus Casatti, 2002	D/N	36.3±16.9	165	MUFAC–IC1216
Plagioscion squamosissimus (Heckel,1840)	–	–	–	MUFAC–IC1224

Claro-García et al. (2013) recorded 94 species in the streams of the Acre basin. However, this species richness was lower than that found at many other sites in the western Amazon basin, including the Purus river, with 112 species being recorded in beach environments (Stewart et al. 2002, Duarte et al. 2010) and 86 species in streams (dos Anjos et al. 2008), 248 species in beach environments of the Negro river (Goulding 1997), 119 species at beaches of the Madeira river (Py-Daniel et al. 2007) and 90 species on the upper Juruá river (Silvano et al. 2001). The relatively low species richness recorded in the present study may be at least partially accounted for by the relatively short period of the study and the use of only one collection method, which is selective of species of reduced swimming capacity (small characids). The use of several alternative collection methods can be essential to guarantee a representative sample of local fish diversity, through the capture of species with distinct swimming capacities found in different environments (Stewart et al. 2002, Duarte et al. 2010). Similarly, a greater sampling effort over a longer period of time may have provided a more reliable sample of local fish diversity, as indicated by the specimens obtained from third parties, which added 20 species to the inventory, almost half the number obtained by trawling. It is important to consider that the variation in

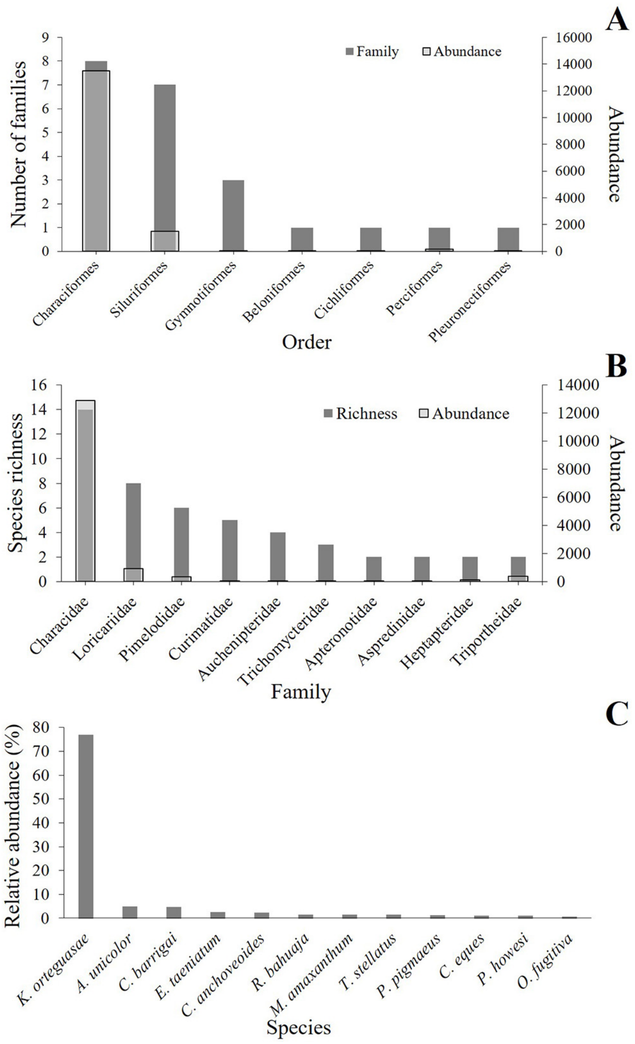

Figure 2. A) The number of families and the abundance of the principal fish orders collected from sandy beaches on the Acre river in the state of Acre, Brazil. B) Species richness and abundance of the different fish families collected from the study area. C) Abundance of the predominant fish species collected from the sandy beaches of the study area.

richness between studies may be due to differences in collection effort and sampling time.

We recorded a predominance of characiform and siluriform species, which is typical of the Neotropical freshwater ichthyofauna (Lowe-McConnell 1999, Reis et al. 2016, Dagosta & de Pinna 2019). As observed in previous studies, the beaches surveyed in the present study were dominated by small characids (Ibarra & Stewart 1989, Jepsen 1997), which reflects their ability to obtain oxygen in the upper layers of the water column, their high trophic plasticity (Abelha et al. 2001), and their ample distribution in the Neotropical region (Jungfer et al. 2013). The considerable abundance of characids, in particular *K. orteguasae* and *C. barrigai*, may be related to their generalist habitat use and high trophic plasticity (Lowe-McConnell 1999, Albert & Reis 2011, Carvalho et al. 2016, Torgler 2016). Trophic plasticity is linked to environmental structure (Abelha 2001), which affects the availability of food, and obliges the species to adapt to different environments, being reflected in the ample feeding spectrum of most teleosts.

Despite a tendency for the stabilization of the species accumulation curve, the regional species pool did not appear to have been sampled as a whole, although the secondary data (specimens donated by third parties) did complement the inventory. Even so, we believe that the true number of species that occur in the beach habitats of the Acre river may be even greater than that recorded here, which reinforces the need for further studies using alternative sampling methods to cover poorly-sampled environments, such as the deepest areas of the river, as well as targeting larger species. The morphology of the fish specimens not identified to the specific level in the present study was incompatible with that of their known congeners, which indicates that these specimens may represent undescribed species. In the past few years, an increasing number of new fish species have been described from all areas of the Amazon basin, and all estimates indicate that many species are yet to be described (Reis et al. 2016, Dagosta & de Pinna 2019, Jézéquel et al. 2020). Even so, the morphological divergences observed in the present study may not necessarily represent new taxa, given that many species are very amply distributed in the Amazon basin, raising the possibility of morphological variation among different populations (Petrolli & Benine 2015; Mateussi et al. 2018).

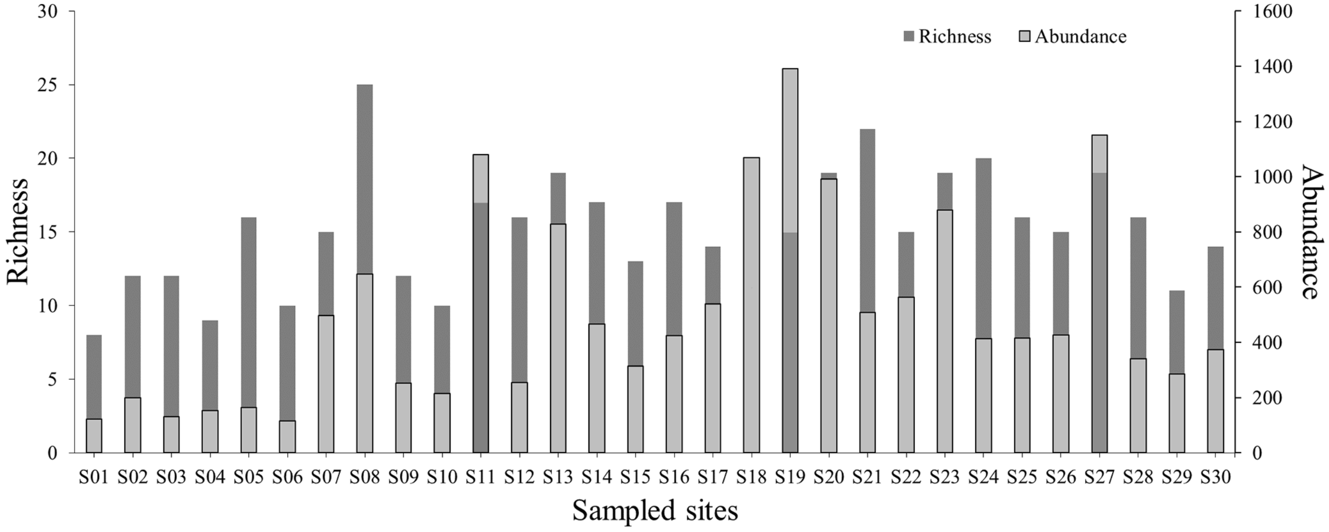

Figure 3. Fish species richness and abundance at the beaches along a 300 km stretch of the Acre river in the state of Acre, Brazil.

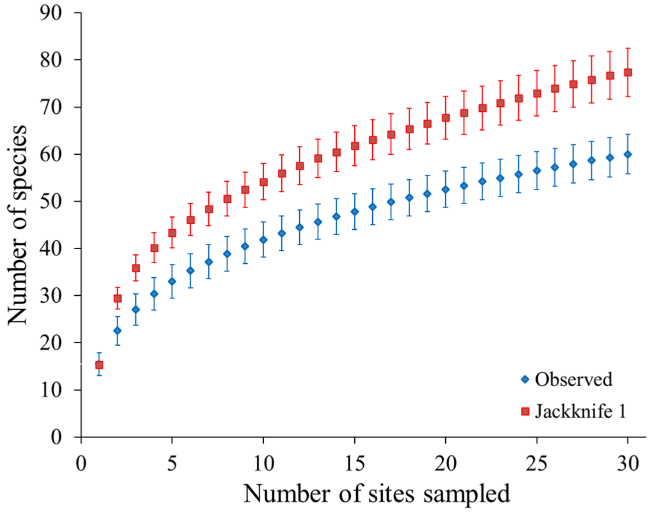

Figure 4. Species accumulation curve of the fish collected from sandy beaches of the Acre river, state of Acre Brazil. The bars represent the confidence interval.

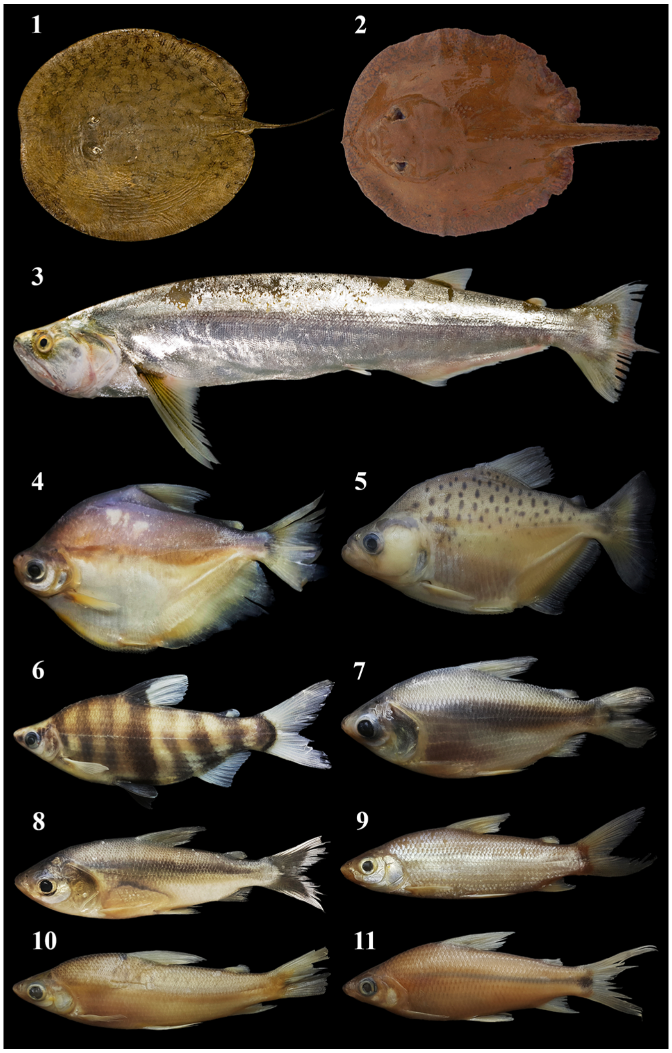

Figure 5. Fish species collected from sandy beaches of the Acre river, Acre, Brazil. **1)** *Paratrygon aiereba*; **2)** *Potamotrygon cf. orbignyi;* **3)** *Rhaphiodon vulpinus*; **4)** *Mylossoma duriventre*; **5)** *Serrasalmus maculatus*; **6)** *Abramites hypselonotus*; **7)** *Psectrogaster amazonica*; **8)** *Psectrogaster rutiloides*; **9)** *Steindachnerina guentheri*; **10)** *Steindachnerina leucisca*; **11)** *Steindachnerina pupula.*

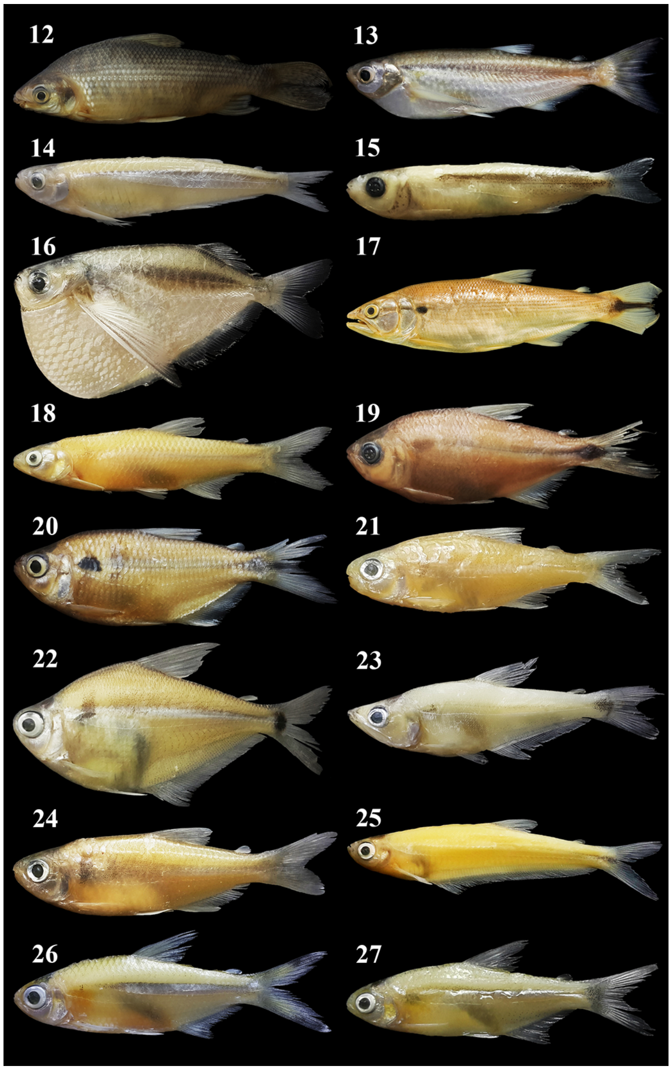

Figure 6. Fish species collected on the beaches of Acre river, Acre, Brazil. **12)** *Prochilodus nigricans*; **13)** *Triportheus albus*; **14)** *Clupeacharax anchoveoides*; **15)** *Engraulisoma taeniatum*; **16)** *Thoracocharax stellatus*; **17)** *Salminus sp.*; **18)** *Aphyocharax pusillus*; **19)** *Astyanax abramis*; **20)** *Astyanax bimaculatus*; **21)** *Creagrutus barrigai*; **22)** *Ctenobrycon spilurus*; **23)** *Galeocharax gulo*; **24)** *Knodus orteguasae*; **25)** *Leptagoniates steindachneri*; **26)** *Moenkausia* sp. "lepidura alta"; **27)** *Odontostilbe fugitive.*

Surveys on the biodiversity of the western Amazon region have enormous potential, not only for the expansion of our knowledge of species distributions, but also the discovery of new species (Corrêa et al. 2018, Silva et al. 2019). In the present study, we collected a specimen of *Micromyzon akamai* Friel & Lundberg, 1996, which is a small fish that buries itself in the substrate to avoid predators (Friel & Lundberg 1996) and is thus collected only very rarely (Ohara & Zuano 2013). This is the first record of *M. akamai* from the state of Acre, and the nearest recorded locality is the main channel of the Madeira river, more than 400 km to the east (Ohara & Zuano 2013). We also recorded *Paratrygon aiereba* (Müller & Henle, 1841), a freshwater stingray not previously known to occur in the Acre basin, with the nearest known localities being on the upper Purus river, approximately 250 km to the northeast (Albert et al. 2012), the Juruá river around 400 km from the Acre river (Silvano et al. 2001), and the Madeira river, approximately 900 km away (Queiroz et al. 2013).

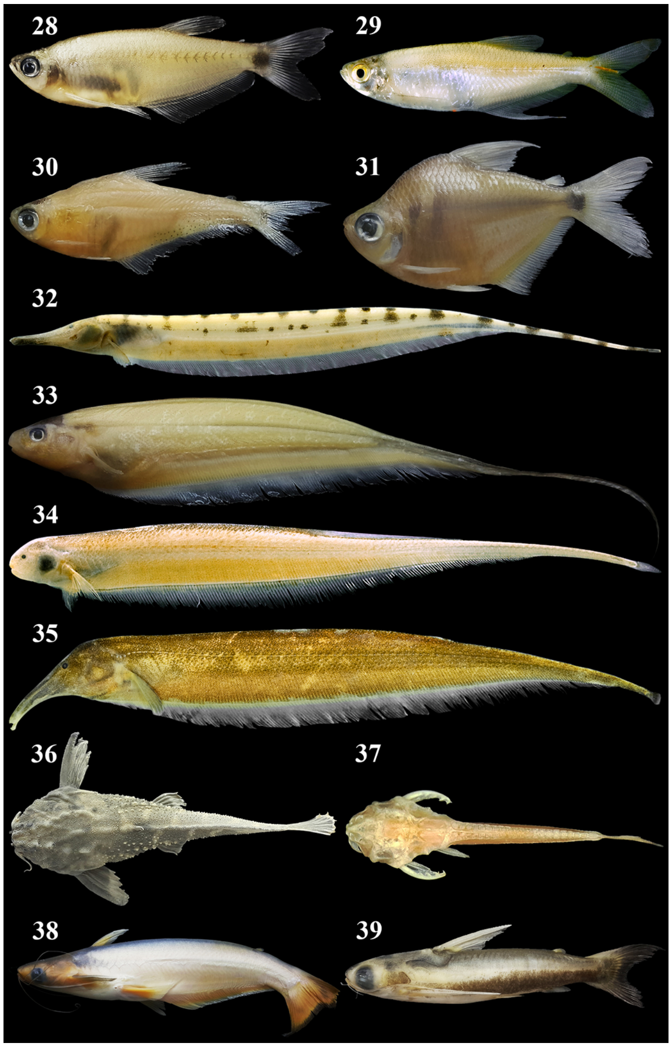

Figure 7. Fish species collected on the beaches of Acre river, Acre, Brazil. **28)** *Paragoniates alburnos*; **29)** *Prionobrama filigera*; **30)** *Protocheirodon pi*; **31)** *Tetragonopterus argenteus*; **32)** *Gymnorhamphichthys hypostomus*; **33)** *Eigenmannia virescens*; **34)** *Sternarchogiton nattereri*; **35)** *Sternarchorhynchus chaoi*; **36)** *Amaralia hypsiura*; **37)** *Micromyzon* cf. *akamai*; **38)** *Auchenipterus nuchalis*; **39)** *Centromochlus heckelii*.

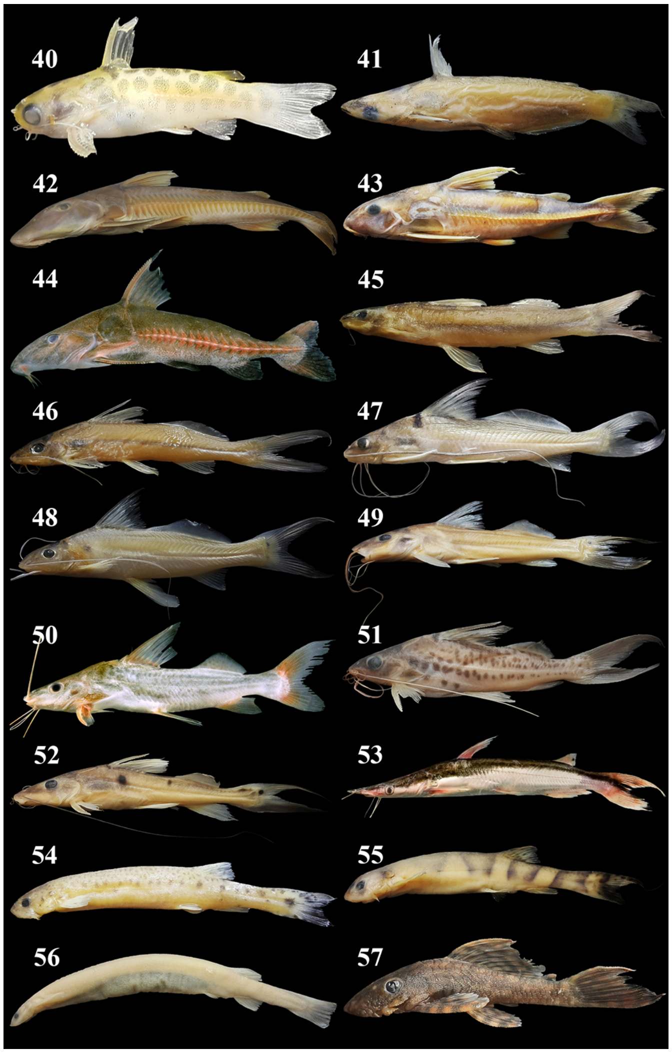

Figure 8. Fish species collected on the beaches of Acre river, Acre, Brazil. **40)** *Centromochlus perugiae*; **41)** *Tympanopleura piperata*; **42)** *Leptodoras acipenserinus*; **43)** *Nemadoras* sp.; **44)** *Oxydoras niger;* **45)** *Imparfinis guttatus*; **46)** *Pimelodella howesi*; **47)** *Cheirocerus eques;* **48)** *Megalonema amaxanthum*; **49)** *Megalonema platycephalum*; **50)** *Pimelodus blochii*; **51)** *Pimelodus* cf. *maculatus*; **52)** *Platysilurus mucosus*; **53)** *Sorubim lima*; **54)** *Henonemus punctatus*; **55)** *Pseudostegophilus nemurus*; **56)** *Vandellia cirrhosa*; **57)** *Ancistrus* sp.

Paratrygon aiereba is currently classified as Critically Threatened in the Red Book of Endangered Brazilian Fauna, and as Data Deficient by the IUCN (Araújo et al. 2018). One of the principal threats to *P. aiereba* is from ornamental fisheries, and while its capture is illegal in Brazil, Colombia and Peru are known to export this species for the ornamental fish trade (Araújo et al. 2018). Demographic studies of *P. aiereba* have shown that its population is declining rapidly and may decrease more than 80% in the near future (Araújo et al. 2018). In addition, Frederico et al. (2012) found genetic variation among populations that may be evidence of a species complex (Carvalho et al. 2003, Rosa et al. 2010). Given this, further research and local conservation initiatives are urgently needed for this species.

Studies of Amazonian fish have found a greater species diversity in beach habitats than in other aquatic systems, such as lakes, streams, and floating vegetation (Goulding 1997, Py-Daniel et al. 2007). This is due to the physical and structural characteristics of beach environments, such as their slower currents, transparency, and depth, which result in an abundance of both small species and the juveniles of larger species. These fish use the shallow waters of the beach habitat to shelter from predators, such as large catfish, as well as to forage (Lowe-McConnell 1999, Duarte et al. 2010, Olds et al. 2018). Given this, increasing the amount of data available on the fish diversity of sandy beaches will be essential not only for future studies of general biodiversity patterns, but also for the development of effective conservation strategies.

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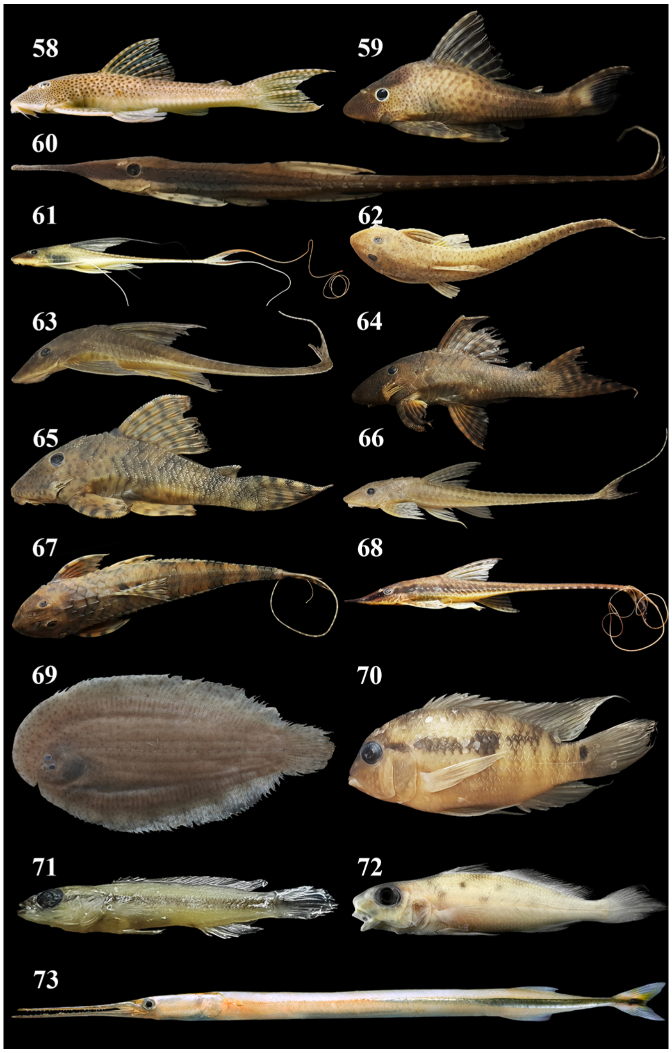

Figure 9. Fish species collected on the beaches of Acre river, Acre, Brazil. **58)** *Aphanotorulus unicolor*; **59)** *Hypostomus* cf. *pyrineusi*; **60)** *Farlowella nattereri*; **61)** *Lamontichthys filamentosus*; **62)** *Limatulichthys griseus*; **63)** *Loricaria* sp.; **64)** *Panaqolus purusiensis*; **65)** *Peckoltia brevis*; **66)** *Rhadinoloricaria bahuaja*; **67)** *Spatuloricaria* cf. *puganensis*; **68)** *Sturisoma lyra*; **69)** *Apionichthys finis*; **70)** *Bujurquina* cf. *syspilus*; **71)** *Crenicichla* sp. "Juvenil"; **72)** *Pachypops pigmaeus*; **73)** *Pseudotylosurus angusticeps.*

Author Contributions

Ronaldo Souza da Silva: Conducted the fieldwork, Identified the specimens in the laboratory, Performed the data analysis and interpreted the results.

Fabiano Corrêa: Ran the data analysis and interpreted the results.

Lucas Pires de Oliveira: Identified the specimens in the laboratory.

Vinicius Guerra: Performed the data analysis and interpreted the results.

Bruno Stefany Barros: Identified the specimens in the laboratory.

Lisandro Juno Soares Vieira: Performed the data analysis and interpreted the results.

Conflicts of interest

The authors declare no competing interests.

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Article

Relative abundances of medium and large mammals in the Cueva de Los Guácharos National Park (Huila, Colombia)

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Abstract: Medium and large mammals are greatly affected by human activities, such as, habitat loss and hunting. In Colombia, these pressures have been most extensive in the dry and Andean forests. However, there is scare information available on the presence or abundance of these organisms for monitoring purposes. This study used photo-trapping cameras to determine the relative abundances of medium and large mammals in a mountain forest in the National Natural Park “Cueva de los Guacharos”, Huila (Colombia). Additionally, we examined whether trail use by humans could affect the distribution of these organisms. The cameras were located every 500 meters along the trails in strategic locations. We obtained the relative abundance of 11 species, being medium-sized mammals the most frequent (*Cuniculus paca, Dasyprocta punctata* and *Mazama rufina*). We recorded the presence of mountain tapir (*Tapirus pinchaque*), dwarf red brocket (*Mazama rufina*) and oncilla (*Leopardus tigrinus*), which are found in a high threat category according to the IUCN. Activity patterns were reported for 4 different species including daytime, nocturnal and cathemeral activities. Finally, we concluded that large mammals avoided trails of frequent use, and although the relative abundance of mammals is not very high, this National Park is still supporting relevant biodiversity.

Keywords: Camera-traps; endangered species; Mammalia; activity patterns.

Abundância relativa dos mamíferos de médio e grande porte no Parque Nacional Cueva de Los Guácharos (Huila, Colômbia)

Resumo: Os mamíferos de porte médio e grande são fortemente afetados por atividades humanas, tais como a perda do seu habitat e a caça. Na Colômbia, essas pressões foram mais extensas em florestas secas e andinas. No entanto, há pouca informação disponível sobre a presença ou abundancia destes organismos para os processos de monitoramento. Este estudo usou câmeras com mecanismos remotos de “armadilhas-fotográficas” (photo-trapping) para determinar a abundância relativa de mamíferos de porte médio e grande numa floresta de montanha no Parque Nacional Natural “Cueva de los Guacharos”, Huila Colômbia. Adicionalmente, examinamos se o uso das trilhas pelos humanos poderia afetar a distribuição destes organismos. As câmeras foram localizadas a cada 500 metros ao longo das trilhas e em locais estratégicos. Obtivemos a abundância relativa de 11 espécies, sendo os mamíferos de médio porte os mais frequentes (*Cuniculus paca, Dasyprocta punctata* and *Mazama rufina*). Se registrou a presença do tapir da montanha (*Tapirus pinchaque*), do veado vermelho (*Mazama rufina*) e do gato-do-mato (*Leopardus tigrinus*), que fazem parte da categoria de espécies em ameaça de acordo com a IUCN. Os padrões de atividade das 4 espécies diferentes foram relatados, incluindo atividades diurnas, noturnas e atividades cathemeral. Por fim, concluiu-se que os grandes mamíferos evitam as trilhas de uso frequente, e embora a abundância relativa de mamíferos não seja muito alta, este parque nacional ainda tem uma biodiversidade proeminente.

Palavras-chave: Câmeras de foto-caça; padrões de atividade; espécies ameaçadas de extinção; Mammalia.

Introduction

The tropical Andes is a region with great biological diversity. Unfortunately, the area is facing great deterioration of the habitat by anthropic intervention, such as, deforestation for agriculture and livestock (Rodríguez et al. 2012, Rodriguez et al. 2013). Currently, conservation strategies in this region are based on national and private reserves, which cover about 10% of the terrestrial area. Andean and sub-Andean forests are the most affected ecosystems, while the paramo is the most protected (Armenteras et al. 2003). Despite these efforts, the situation in national reserves is not optimal, there are high rates of deforestation in National Parks of Colombia (Etter et al. 2006, Miller et al. 2004). For instance, recent years have shown a clear deterioration of the natural areas in the buffer zone of the Cueva de los Guácharos Natural Park (PNNCG) (Hurtado 2013). This phenomenon may be causing problems in population dynamics of organisms that inhabit these areas both by hunting (Roldán and Simonetti 2001) and habitat reduction.

Some medium and large mammals can be considered as keystone species in their ecosystems, since, they play an important role as predators and seed dispersers (Jansen et al. 2012, Terborgh et al. 2001). For example, herbivorous species, as part of trophic networks at intermediate levels, can affect plant populations and control densities of other species, as predators (Terborgh & Wright 1994, Kelly & Holub 2008, Lizcano & Cavelier 2004). Its effect can be so great that it is considered possible that there is a direct relation in the floristic composition of the forest in future years, when the density or presence of certain mammal species in the area changes (Roldán & Simonetti 2001). These effects are difficult to measure and depend on the population density (Stevenson 2011).

There are few cases of mammal hunting in Colombia's national parks. Fortunately, no massive logging or crop events were identified for more than 30 years in Cueva de Los Guacharos National Park (UAESPNN 2005), which is our study site. However, given the continuous advance of the agricultural frontier in the surroundings of the park, we should consider a possible increase on hunting pressure for the coming years, generating displacement of individuals by the deterioration of their habitat. For these reasons, it is of great importance to know the current status of the populations of mammals. Studies of relative abundance are very important for the detection of changes in wildlife population dynamics (Lyra-Jorge et al., 2008). This information can be used to make decisions about management and conservation of a specific population or area. According to the management plan of the PNNCG, within and at its surroundings there are 59 species of mammals, belonging to 11 orders and 25 families. The orders with the greatest relative abundance of species are Chiroptera, Carnivora and Rodentia (UAESPNN 2005), mostly small individuals, which are not the focus of this study. In terms of large and medium-sized mammals, there is a list of species such as the mountain tapir *(Tapirus pinchaque),* the spectacled bear (*Tremarctos ornatus*), the dwarf red brocket (*Mazama rufina*) and the northern pudu (*Pudu mephistophiles*) most of them at risk of extinction (UAESPNN 2005). However, some of these species, such as the mountain tapir, has not been recorded in recent years. The threat status of the different species presented in the management plan of the PNNCG was obtained from the International Union for Conservation of Nature's Red List of Threatened Species better known as the IUCN red list (IUNC, 2005). This is the world's most comprehensive information source on the global extinction risk status of different species in the world. It provides information about range, population size, habitat and ecology, threats and conservation actions for a vast number of species worldwide. Thus, the IUCN red list is an important tool to make informed conservation decisions.

The study of terrestrial mammals is a difficult and laborious task. Since, they are elusive animals with large ranging areas, and usually difficult to observe or follow (Yasuda 2004). For these reasons tracking techniques such as footprints and direct observation have been used on studies of relative abundance and distribution of medium and large mammals (Olifiers et al., 2011). However, in some cases the determination of tracks is ambiguous (Emmons 1997). In consequence, it is very useful to use monitoring techniques that make identification at the species level easier (Yasuda 2004). Monitoring mammals using camera traps is one of the most successful, non-invasive methods that includes identification of species with cryptic habits (Silveira et al., 2003, Treves et al., 2010). This method with a proper sampling design can provide detailed and continuous information on the state of the populations (Ahumada et al., 2011, Silveira et al., 2003). For some years, the use of camera traps has allowed the estimation of mammalian populations, being more commonly used in felids (Soria-Diaz et al., 2010, Silver et al., 2004, Wang & Macdonald 2009).

For this study it is argued that human presence, even if they are not hunters, affects the habitat use of mammals. According with this idea, it is expected to find higher rates of sighting on trails with little human use, at least for large mammals, since these tend to avoid places with anthropic activity. In addition, the information generated in this study is expected to become a reference for conservation measurements at the national park.

Materials and Methods

1. *Area of study*

The study was carried out in Cueva de los Guácharos National Park (PNNCG), located in the departments of Huila and Caquetá (Colombia) 1º 36'56.0 '' N, 76º06 '08.5' 'W. Total area is 9,000 ha, with elevations between 1700 and 3000 masl. The park has an annual average temperature of 16º C with an annual average rainfall of 3,100 mm and a relative humidity of 87%. In 1980, UNESCO declared it a Biosphere Reserve (UAESPNN 2005).

2. *Setting of the cameras*

A total of 18 camera traps (Cuddeback Capture model) were used. These operated 24 hours a day, and were programmed with an interval of 30 seconds between events. The cameras were set at a distance of approximately 500 m between them and were located in existing trails taking into account the use of these to see if there were differences between places with greater human traffic (n = 8 cameras) and trails that were not used frequently (n = 9) defined as those who have less than a weekly visit by officials, visitors or researchers. In addition, a camera was placed in a cave inhabited by oilbirds (*Steatornis caripensis*), which is a very busy place visited by tourists (n = 1). In this place colonies of oilbirds discharge many seeds, which can be a mammalian food resource. Each of the cameras was installed at a height of 40 cm from the ground. The study was carried out between elevations of 1940 and 2340 masl. The cameras were checked every two months by changing the batteries and memory card for a total of 14 months of sampling.

3. *Data analysis*

When analyzing the obtained photos, each animal occurrence was taken as a sighting, except photos of the same species taken with a difference of less than 30 minutes, which were taken as a single sighting, since the probability of capturing the same individual is very high (O'Brien 2010). A medium-sized mammal was considered to have a weight between 250 g and 4 kg, while large mammals were considered greater than 4 kg. With the obtained images, we estimated the relative abundances of each mammal species. The relative abundance of mammals was calculated by taking into account the number of times an individual was recorded and assuming that all individuals are equally likely to be recorded (Springer et al., 2012), as well as the total number of night traps (O'Brien 2010).

$$\text{Encounter Index}(I) = \left(\frac{\text{number of records of the species}}{\text{number of nights trap}}\right) \quad (1)$$

$$\text{Relative abundance per night trap}(AR1) = \text{I} * 100 \quad (2)$$

Species were classified as diurnal, nocturnal or cathemeral (van Schaik & Griffiths 1996). Activity patterns were estimated only for species that were recorded at least eleven independent times (Monroy-Vilchis et al., 2011). Graphs were made in the Oriana program, which allow seeing the patterns of activity in a circular way (Kovach Computing Services 2004). Statistical tests Mann-Whitney and Kruskal-Wallis obtained using R software (R-Development-Core- Team 2008), were used to evaluate statistical differences between trails with different frequencies of use.

Results

With a total of 3595 night traps, 176 records of mammal belonging to 11 species were obtained (Table 1). Within the list of species, and according to the IUCN criteria some of the species found were at high threat level such as the mountain tapir (*Tapirus pinchaque*), which was in the endangered category. Other mammals such as the spectacled bear (*Tremarctos ornatus*), the oncilla (*Leopardus tigrinus*) and the dwarf red brocket (*Mazama rufina*) with vulnerable category (VU) were also registered. The rest of the registered species were in the least concern category (LC).

Obtained relative abundances showed higher values for medium mammals such as *Cuniculus paca* and *Dasyprocta punctata*, followed by large mammals, with species such as *M. rufina*, *Puma concolor* and *T. ornatus* (Fig. 1). For relative abundance recorded in the different type of trails, it was observed that; taking into account all species recorded in the study there was no significant differences (Mann-Whitney: U = 2000, P = 0.228). However, by discriminating by size category, the results suggest that only large mammals were sensitive to the use of trails by humans (M-W: U = 0.0, P = 0.049). Median mammals do not showed statistically significant results (M-W: U = 36.50, P = 0.7341).

Table 1. Species registered by trap-cameras in Cueva de Los Guacharos National Park (Colombia), including number of records, the IUCN threat category, encounter rate, relative abundance, and the type of trail where they were found (F trails of frequent use by humans and NF trails with less use).

Family Species	Local name	Records	IUCN	I	AR 1	Trail type
Sciuridae						
Notosciurus granatensis	ardilla	3	LC	0,0008	0,08	F-NF
Cuniculidae						
Cuniculus paca	boruga	46	LC	0,0128	1,28	F-NF
Cebidae						
Sapajus apella	maicero	1	LC	0,0003	0,03	NF
Tapiridae						
Tapirus pinchaque	danta	2	EN	0,0006	0,06	NF
Didelphidae						
Didelphis marsupialis	fara	4	LC	0,0011	0,11	F
Procyonidae						
Nasua nasua	cusumbo	8	LC	0,0022	0,22	NF
Dasyproctidae						
Dasyprocta punctata	ñeque	44	LC	0,0122	1,22	F-NF
Ursidae						
Tremarctos ornatus	oso	8	VU	0,0022	0,22	NF
Felidae						
Puma concolor	puma	19	LC	0,0053	0,53	F-NF
Leopardus tigrinus	tigrillo	1	VU	0,0003	0,03	NF
Cervidae						
Mazama rufina	venado	40	VU	0,0111	1,11	NF

The difference of each trail individually did not present significant statistical differences (Kruskal-Wallis = 9.2, P = 0.13).

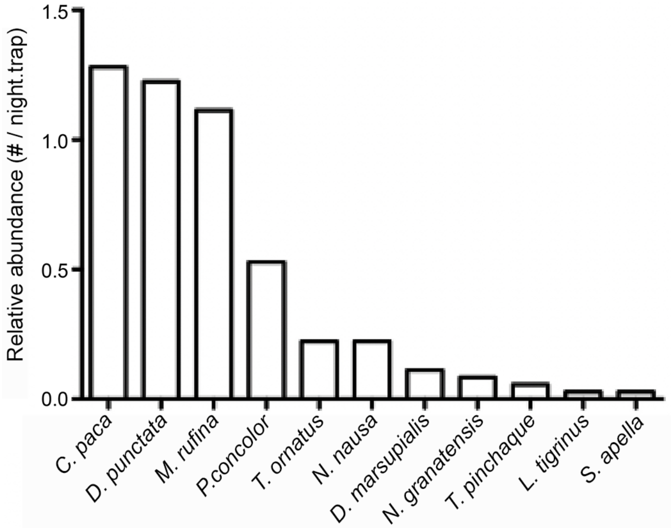

Figure 1. Relative abundance for each species.

Activity patterns for four species were found, including diurnal *(D. punctata*), nocturnal (*C. paca*), and cathemeral *(P. concolor* and *M. rufina*) (Fig. 2).

Discussion

The use of the trails by humans affected the distribution of large mammals, since they were detected less frequently in commonly used trails. The puma *(P. concolor*) proved to be the only large mammal recorded on transit trails. Although the park is in a good state of conservation, it is important to maintain a large buffer area, as it has been observed that the greater the fragmentation the lower density of mammals inhabit a place (Ahumada et al., 2011).

The proportion between large and medium mammals is expected since it coincides with previous findings where medium mammals such as *C. paca* and *D. punctata* tend to show higher relative abundances than other species such as predators (Arcos 2010). Median mammals reported are in many cases the potential prey of large carnivorous mammals, such as *P. concolor* (Hernández-Guzmán et al., 2011).

When comparing results with the ones reported in bibliography, this study indicate one of the places with the lowest number of species

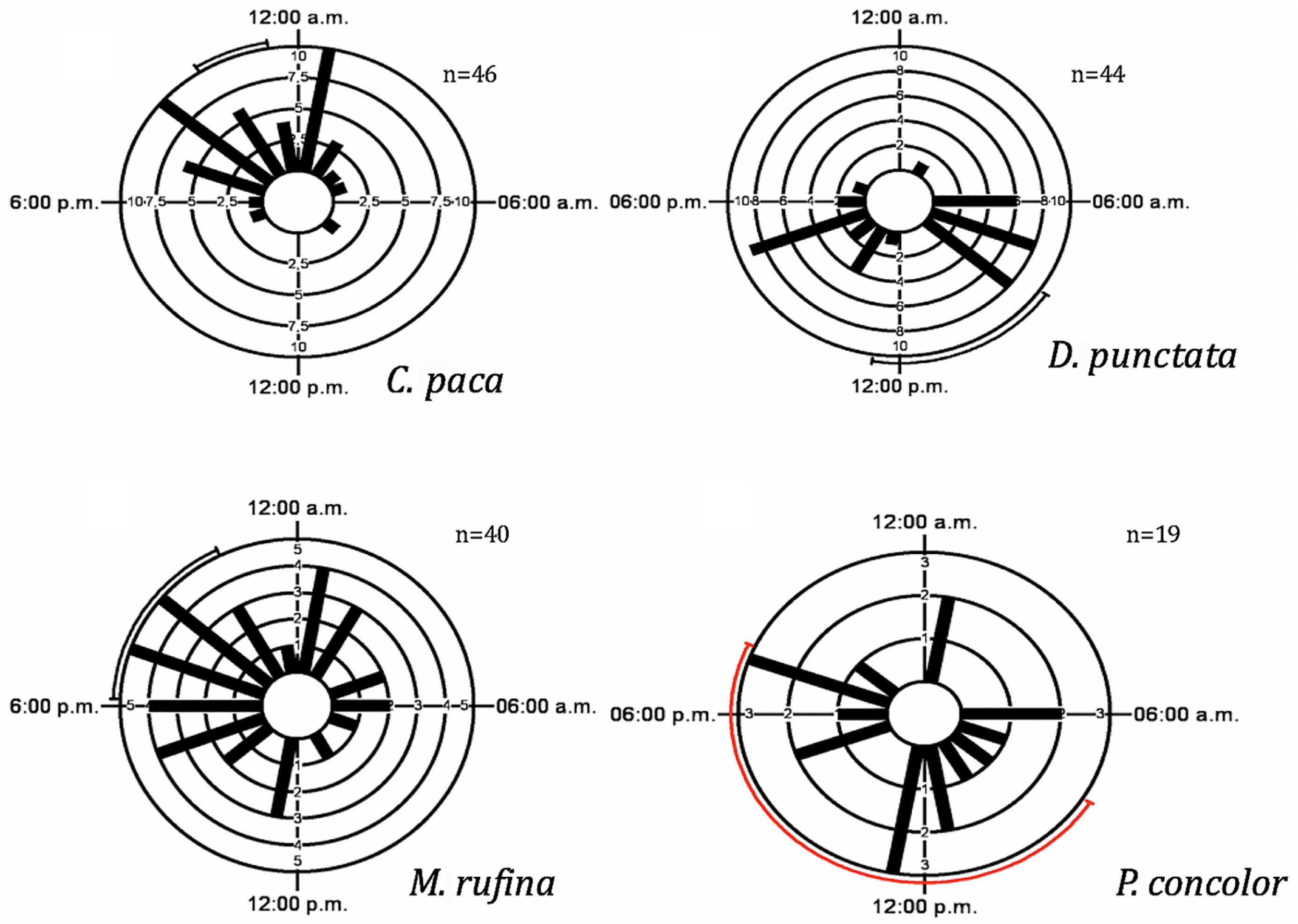

Figure 2. Activity patterns of mammal species showing at least 7 sightings in Cueva de Los Guacharos National Park (Colombia). a: lowland paca (*C. paca*), b: agouti (*D. punctata*), c: dwarf red brocket (*M. rufina*), d: puma (*P. concolor*). The red line in the graph of puma indicates no specific trend for the hours in which this is active.

(Lira-Torres & Briones-Salas 2012, Botelho et al., 2012, Ahumada et al., 2011). It is important to realize that most of the studies have been carried out in lowland forests, which have higher productivity, and greater number of species. Similar diversity and abundance of species are reported in the Andes mountain range, for example in Ecuador (Arcos, 2010).

Regarding to the activity of different species, the lack of a clear pattern in the activity of the puma (*P. concolor*), which can have daytime and nighttime activity, could indicate a good state of conservation of the area, since in places with greater human and hunting presence, these tend to decrease their diurnal activity (Paviolo et al., 2009). In the same way, it is possible to observe how the periods of more records coincide with the activity peaks of some species that are common prey of the puma, such as *M. rufina*, *C. paca,* and *D. punctata*. As previously mentioned, the abundance of these prey species is higher than that of the predator (Foster et al., 2013).

The finding of the mountain tapir (*T. pinchaque*) and the spectacled bear *(T. ornatus*) is a very positive fact, since these species are highly affected by hunting. Their densities tend to be small, being the tapir one of most affected by this threat (Cavelier et al., 2011). Tapirs have shown an important role as herbivores and some consider them important seed dispersers, which help to infer that the area is in a good state of conservation (Downer, 2001).

We conclude that the use of trails by humans can affect the presence of large mammals, something to consider when choosing conservation and study areas. The PNNCG has priority species for conservation. Although the relative abundances of some of these, such as the paramo tapir, are low, their presence is an indicator that the park still has a good state of conservation, since in general these are individuals very sensitive to anthropic activities. The PNNCG has been open to scientific research; however, this was the first study using camera traps, which allows to complement the catalog of fauna that has been observed. In addition, information on the relative abundance of terrestrial mammals may allow future monitoring. We hope that these data can be useful in the elaboration of conservation actions, management plans and other scientific studies.

Supplementary material

The following online material is available for this article:

Appendix - Photographs of some of the species recorded during the study in Cueva de Los Guacharos National Park (Colombia).

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Author Contributions

Felipe Gast: Substantial contribution in the concept and design of the study. Contribution to data collection. Contribution to data analysis and interpretation. Contribution to manuscript preparation. Contribution to critical revision, adding intellectual content.

Pablo R. Stevenson: Substantial contribution in the concept and design of the study. Contribution to data analysis and interpretation. Contribution to manuscript preparation. Contribution to critical revision, adding intellectual content.

Conflicts of Interest

The authors declare that they have no conflict of interest related to the publication of this manuscript.

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Short Communication

The use of dead stand trees by birds: a prospective study in a Brazilian hydroelectric dam

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Abstract: Hydropower constructions cause severe disturbances to biodiversity. Arboreal plants die after flooding; however, they expose their emerged branches (also called paliteiros) as a new habitat for animals and plants. Nothing is known about how paliteiros influence the presence of aquatic birds. This study verified which species of birds use the paliteiros, what are their behaviors and probably consequences to ecological processes. In November 2018, we sampled 5 km of paliteiros recording perched birds and their behaviors in the Três Marias dam, a 1040 km^2 flooded area in southeastern Brazil. In total, 5.4% of paliteiros were used by 14 bird species. The Neotropic Cormorant *Nannopterum brasilianus* (Gmelin, 1789) was the most frequent species. Birds mainly preened feathers; they also rested (with no apparent activity), defecated, hunted, fed, vocalized and drank water. Attention must be focused to some behaviors such as resting and defecation, which may influence long-term ecological processes (e.g., incorporating additional organic matter and changing aquatic community) in the dam.

Keywords: *Behavior; Birds; Hydropower building; Human impact.*

O uso de paliteiros por aves: um estudo prospectivo em uma usina hidrelétrica no Brasil

Resumo: Construções de hidrelétricas causam severos distúrbios à biodiversidade. Após a inundação de grandes áreas as plantas arbóreas morrem, apesar disso, elas deixam expostos seus galhos emersos (também chamados de paliteiros) criando um novo hábitat para animais e plantas. Nada se sabe sobre como os paliteiros influenciam a presença de aves aquáticas. Este estudo verificou quais espécies de aves utilizam os paliteiros, quais comportamentos elas exibem e suas prováveis consequências para processos ecológicos. Em novembro de 2018 foram amostrados 5 km de paliteiros, as aves empoleiradas nestas estruturas e seus comportamentos na represa de Três Marias, uma área inundada de 1040 km^2 no sudeste do Brasil. Ao total, 5.4% das árvores mortas foram utilizadas por 14 espécies de aves. O biguá *Nannopterum brasilianus* (Gmelin, 1789) foi a espécie mais frequente nos paliteiros. As aves frequentemente limparam as penas, descansaram (sem nenhuma atividade aparente), defecaram, caçaram, se alimentaram, vocalizaram e beberam água. Mais atenção deve ser focada em alguns comportamentos das aves tais como o descanso seguido de defecação que podem influenciar processos ecológicos a longo prazo (p. ex., a incorporação de matéria orgânica e mudança de comunidade aquática) na represa.

Palavras-chave: *Aves; comportamento; construção de hidrelétricas; impacto humano.*

Introduction

Human impacts tied to the exponential increase of urbanization are considered major forces to the loss of biodiversity (McDonald et al. 2013). The construction of dams to generate electricity is a widespread example of impact that converts natural areas to artificial areas. It emerged by manipulation of natural water courses and creation of reservoirs which can cause serious disturbances to biodiversity (Stanford & Ward 1992, Forsberg et al. 2017). Many terrestrial animals and plants are excluded from local community after flooding of large areas due to habitat loss (Báldi et al. 1998, Mallik & Richardson 2009). Despite it, damming also results in the appearing of new habitats such as remaining dead and standing trees called "paliteiros". In many cases, some species of birds may profit in dams by the changes promoted by humans and individuals within population may adjust to disturbances of their environment (Reitan & Thingstad 1999). Therefore, aquatic birds can use paliteiros as a perching substrate, however, nothing is known about their frequency of use and behaviors that they exhibit.

A similar and parallel example of remaining perching structure to birds are the scattered trees in human-modified terrestrial landscapes that are useful to species as resting and feeding places during movements between areas serving as stepping stones (Manning et al. 2006). Frugivorous birds can rest or feed in scattered trees or artificial perches and they can defecate and regurgitate seeds contributing to natural regeneration of degraded areas. Also, perches are used by birds to hunt other organisms like ants and rodents thus contributing to a balanced food chain (Herrera & Garcia 2009, Guidetti et al. 2016, Vogel et al. 2018).

The Brazilian territory is privileged in watersheds and it is the second country that generates more hydropower in the world (behind China), with hydroelectricity totaling 68.1% of the country's electricity and more than 290 large dams (Oliveira 2018). Although the negative perspective on low biodiversity found after construction of hydroelectric dams (Batista et al. 2012), no knowledge exists of what concerns the influence of dead standing trees as perching structures for birds in flooded areas and its ecological importance. Here, we verified which species of birds use the paliteiros in the Três Marias dam located in southeastern Brazil. We also recorded what behaviors birds exhibit and if the quantity of dead branches in paliteiros may influence their momentary presence in the flooded area. With this study, we expect to raise initial information about the use of paliteiros by birds and their ecological consequences in dams.

Methods

1. *Study area*

The study was conducted at Pirapitinga Ecological Station (PES), which has an approximate area of 1,090 ha and 20 km perimeter. It is located in the reservoir of Três Marias hydroelectric power in the municipality of Morada Nova de Minas, Minas Gerais State, southeast Brazil. The PES is located at the confluence of the Riachão stream with the São Francisco river (18°20' S - 18°23' S and 45°17' W - 45°20' W) (Azevedo et al. 1987)(Figure 1). It is surrounded by a high amount of paliteiros resulted from dead savanna or Cerrado trees caused by the flooding of 1962. The paliteiros emerged 6-7 years ago due to the reduction of the water level of the dam. There are 36 species of aquatic birds in the PES area (Plano de Manejo Estação Ecológica de Pirapitinga 2013).

2. *Data collection*

Data collection was conducted by a team of five people in the morning from 07:30 h to 12:00 h on 26 November 2018. Approximately

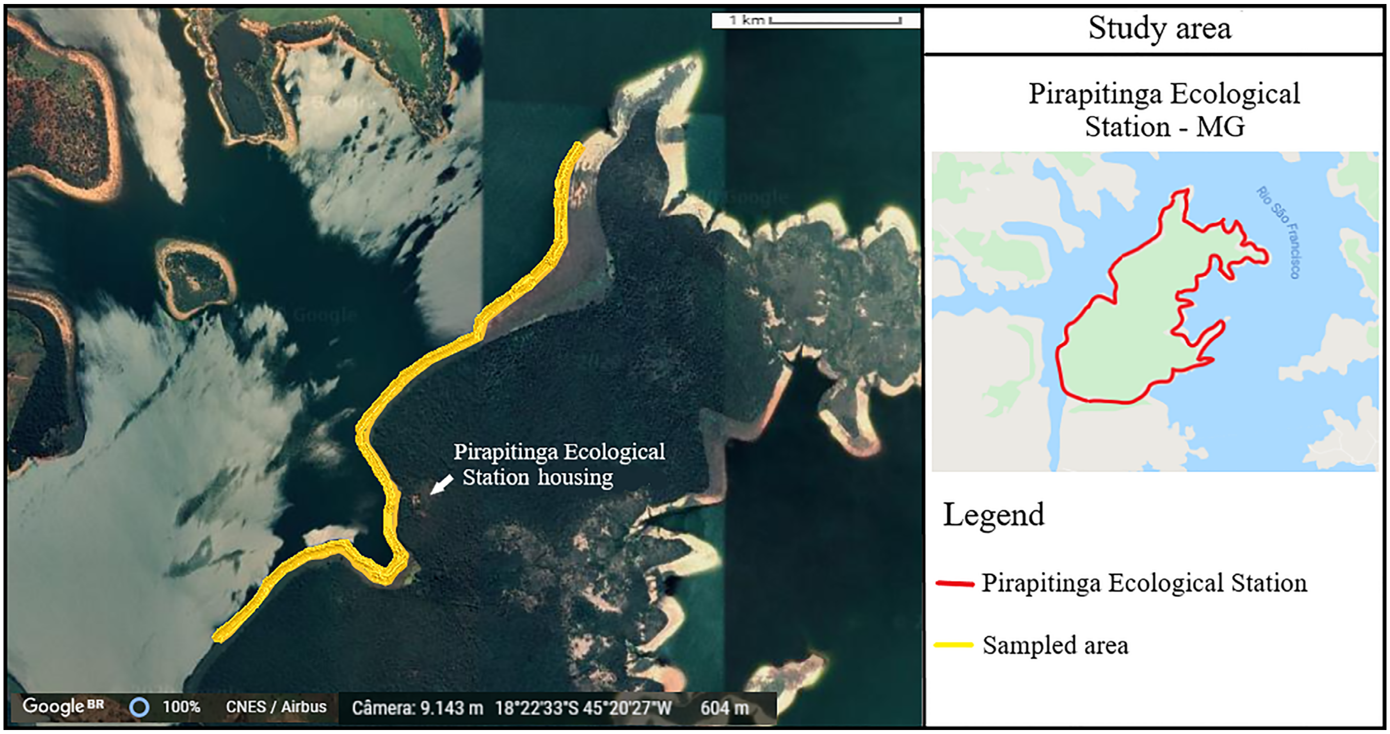

Figure 1. Location of the study area. Paliteiros were sampled in the border of Pirapitinga Ecololgical Station, southeastern Brazil.

5 km of paliteiros were sampled at the edge of the main channel from Três Marias dam, near the PES housing. We sampled the abundance of dead trees in paliteiros, the bird species richness and the frequency of use of paliteiros by birds as well as their behavior when perched in an emerged branch (or tip). Different portions of paliteiros were sampled simultaneously to avoid double counting of the same individual bird. We treated one paliteiro as a total of 100 tips of emerged branches (i.e., above water surface) which corresponds to 33 ± 8.30 dead trees (see results). Some of the branches were bifurcated and contained more than one tip. Other future studies may differently deal a paliteiro unit than us considering that there is no previous definition of how many trees or tips form a paliteiro unit. Perched birds were identified, counted and observed with the aid of binoculars. The focal animal technique was applied for 30 seconds per bird and bird behavior was identified and quantified. We classified bird behavior into nine categories: (1) resting, when perched birds apparently exhibited no active behavior; (2) preening, when birds cleaned feathers with beak or feet; (3) hunting, when birds flew or dove to chase a prey; (4) feeding; (5) drinking; (6) defecating; (7) communicating, when birds vocalized; (8) nesting, when birds had a nest or were building a nest; and (9) drying feathers with opened wings. The nomenclature of the species follows the Brazilian Ornithological Records Committee (Piacentini et al. 2015).

3. *Data analyses*

Jackknife 1 estimator was used to estimate the number of bird species in paliteiros. We also used a logistic regression to know if the number of dead trees (i.e., sampling units) in paliteiros influenced the presence/absence of birds. An emerged individual branch (i.e., with one tip) or an emerged and bifurcated branch (i.e., with two or more tips) is the closest way of what we found to represent a dead tree. This procedure was taken because we did not have access to the submerged and complete structure of branches and stems to accurately isolate individual trees in the dam.

The species accumulation curve with rarefaction method analysis was used to know the representativeness of bird species considering the number of paliteiros and number of birds sampled, respectively. The same procedure was adapted to know the representativeness of bird behavior types. In this case, we considered types of behavior instead specific species. Analyses were performed in R Statistical software v 3.5.1 (R Core Team 2019). "Vegan" was the complementary R package used in the analyses.

Results

We sampled 28 paliteiros in PES. Each paliteiro has 33 ± 8.30 dead trees (maximum and minimum number of trees were 65 and 20, respectively). Seventy-three birds of 14 species used 5.4% (N = 48) of the total number of dead trees (N = 888) in paliteiros. The Neotropic Cormorant *Nannopterum brasilianus* (Gmelin, 1789) was the most frequent species (Figure 2). The number of dead trees did not influence the presence of birds in paliteiros (df = 26; z = -0.54; p = 0.59). Jackknife 1 estimated 20.75 ± 2.55 (SE) species of birds to the sampled area. Rarefaction method analysis estimated the need of four paliteiros (or 400 tips or nearly 12 dead trees) or 10 individuals of birds to record an additional species of bird.

Preening was the most frequent behavior used by birds followed by resting (Figure 3, Table 1). No nesting or wing-drying behaviors were recorded. Rarefaction method analysis estimated the need of eight paliteiros (or 800 tips or nearly 24 dead trees) or 16 individuals of birds to record a new type of behavior.

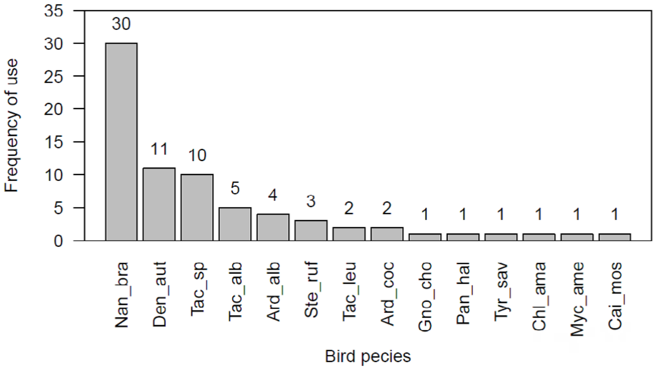

Figure 2. Bird species and their frequency of use of paliteiros in Três Marias dam, southeastern Brazil. Numbers above bars evidence the frequency of use of paliteiros by each bird species. Nan_bra: *Nannopterum brasilianus*; Den_aut: *Dendrocygna autumnalis*; Tac_sp: *Tachycineta* sp.; Tac_alb: *Tachycineta albiventer*; Ard_alb: *Ardea alba*; Ste_ruf: *Stelgidopteryx ruficollis*; Tac_leu: *Tachycineta leucorrhoa*; Ard_coc: *Ardea cocoi*; Gno_cho: *Gnorimopsar chopi*; Pan_hal: *Pandion haliaetus*; Tyr_sav: *Tyrannus savanna*; Chl_ama: *Chloroceryle amazona*; Myc_ame: *Mycteria americana*; Cai_mos: *Cairina moschata*.

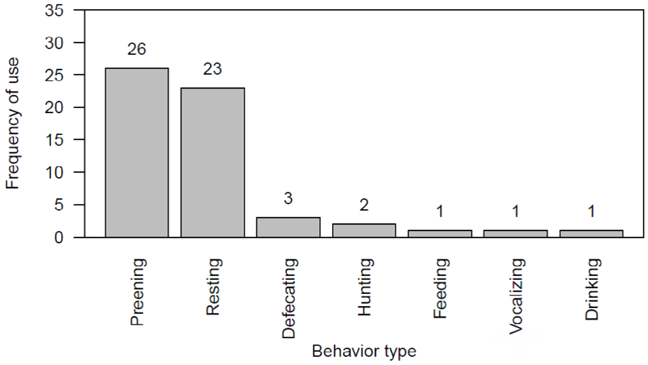

Figure 3. Types of bird behavior and their frequency of use in paliteiros from Três Marias dam, southeastern Brazil. Numbers above bars evidence the frequency of behavior.

Discussion

The present study was the first to evaluate the use of paliteiros by birds. Twelve out of 14 species were aquatic birds and they represented 33.3% of a total of 36 species of aquatic birds in the entire region (Plano de Manejo Estação Ecológica de Pirapitinga 2013). The Jackknife estimator pointed that no more than 21 species or 58% of total species may use paliteiros in the region. Also, birds showed a variety of behaviors when perched in paliteiros. Although the short-time nature of our results, we sampled a significant number of dead trees and this prospective study incorporated a sampling protocol that can be used in future researches regarding the use of paliteiros by birds. Furthermore, we argue that more attention also must be focused to some behaviors

Table 1. Bird species and their behaviors when perched on paliteiros in Três Marias dam, southeastern Brazil. (P) preening, (R) resting, (D) defecating, (H) hunting, (F) feeding,(V) vocalizing, and (Dr) drinking. Number in parenthesis indicates the frequency of behavior type.

Taxa	Portuguese name	English name	Behavior
Anseriformes Linnaeus, 1758			
Anatidae Leach, 1820			
*Dendrocygna autumnalis (*Linnaeus, 1758)	marreca-cabocla	Black-bellied Whistling-Duck	R(4) , D(1), P(2), V(1)
*Cairina moschata (*Linnaeus, 1758)	pato-do-mato	Muscovy Duck	P(1)
Ciconiformes Bonaparte, 1854			
Ciconiidae Sundevall, 1836			
Mycteria americana Linnaeus, 1758	cabeça-seca	Wood Stork	R(1)
Suliformes Sharpe, 1891			
Phalacrocoracidae Reichenbach, 1849			
Nannopterum brasilianus (Gmelin, 1789)	Biguá	Neotropic Cormorant	R (5), D(2), P(8), Dr(1)
Pelecaniformes Sharpe, 1891			
Ardeidae Leach, 1820			
Ardea cocoi Linnaeus, 1766	garça-moura	Cocoi Heron	R(2)
Ardea alba Linnaeus, 1758	garça-branca	Great Egret	R(3), H(1), P(1)
Accipitriformes Bonaparte, 1831			
Pandionidae Bonaparte, 1854			
Pandion haliaetus (Linnaeus, 1758)	águia-pescadora	Osprey	P(1)
Coraciiformes Forbes, 1844			
Alcedinidae Rafinesque, 1815			
Chloroceryle amazona (Latham,1790)	martim-pescador-verde	Amazon Kingfisher	R(1), H(1), F(1)
Passeriformes Linnaeus, 1758			
Tyranninae Vigors, 1825			
Tyrannus savana Daudin, 1802	Tesourinha	Fork-tailed Flycatcher	P(1)
Hirundinidae Rafinesque, 1815			
Stelgidopteryx ruficollis (Vieillot, 1817)	Andorinha-serradora	Southern Rough-winged	R(1), P(2)
Tachycineta albiventer (Boddaert, 1783)	andorinha-do –rio	White-winged Swallow	R(1), P(3)
Tachycineta leucorrhoa (Vieillot, 1817)	andorinha-de-sobre-branco	White-rumped Swallow	R(1), P(2)
Tachycineta sp.			R(4), P(4)
Icteridae Vigors, 1825			
Gnorimopsar chopi (Vieillot, 1819)	pássaro-preto	Chopin Blackbird	P(1)

(e.g., defecation, and hunting) that may influence ecological processes in dams.

The water level of the Três Marias dam has been reduced and exposed the dead arboreal trees 6-7 years ago. Although the number of dead trees did not influence the presence of birds in paliteiros, a gradual process of colonization and use of paliteiros by aquatic bird species should be occurring in the area. During the colonization process, several adaptations (e.g., life history, general tolerance to disturbances, ability to disperse to other habitats) that are specific for species affect the possibilities for each bird to adjust (or not) its behavior in human-altered conditions. For instance, the Neotropic Cormorant (*N. brasilianus*), a top aquatic predator, was the most frequent species in our study and it may be considered as an example of generalist species with high incidence of colonization in anthropic environments (Orta 1994, Sick 1997). The cormorants showed a bunch of behaviors in the paliteiros and they opportunistically hunts a variety of prey using submerged and efficient dives in small and large natural and artificial lakes, as well as in rivers and marine ecosystems throughout the Brazilian territory (Barquete et al. 2008). The presence of cormorants and other aquatic top predator birds (e.g., heron and kingfishers) may alter significantly their prey abundance (Steinmetz et al. 2003).

Some birds that defecated in paliteiros may have acted as fertilizer agents incorporating additional organic matter into water and/or acting as food provider to detritivorous species (e.g., fish and crustaceans). Therefore, birds may play an important role as nutrient vectors (mainly phosphorus and nitrogen) which interfere in the trophic chain of aquatic ecosystems (Manny et al. 1994). This is a similar mechanistic process that occurs when frugivorous birds defecate seeds from perches in degraded areas: they may feed other organisms (e.g. ants), increase the seed bank, and indirectly modify the local community of plants and animals (Christianini & Oliveira 2009, Herrera & Garcia 2009, Guidetti et al. 2016). Furthermore, some usual bird places such as dormitories and rookeries may be more protected from predators when they border aquatic ecosystems similarly to paliteiros; the high concentration of individuals (including their droppings) may change local macrophytes biomass and their associated organisms (Castelo-Branco 2008). More studies are necessary to investigate if paliteiros facilitate or attract higher concentration of other organisms such as plants, fish and invertebrates.

The use of paliteiros by birds is a recent and underexploited theme. Our study is far from a definitive conclusion, however, it is the first step to discover more information about the importance of this man-altered habitat to birds and how the presence of birds may positively or negatively influence ecological processes. We encourage more comparative studies to verify if older flooded areas with paliteiros may have higher bird biodiversity than recent ones and if birds may comprise more specific ecological functions in the local food chain.

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Author Contributions

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Contribution to data analysis and interpretation: C. Cestari.

Contribution to the writing of the paper: A.C. de Oliveira, E. Centeno, J.R. Assis, P.M.S. Bomfim, C. Cestari.

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Short Communication

An Unprecedented Record of Parasitoidism of Formicidae by a Sarcophagid Fly

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Abstract: The first case of parasitoidism of a Myrmicinae ant by a sarcophagid fly has been observed in the Brazilian Cerrado. The larva of a *Helicobia* Coquillet (Sarcophagidae) specimen fed on head tissues of an *Atta laevigata* Smith, 1858 soldier and an adult male fly emerged from a puparium formed outside the host.

Keywords: *Brazilian savanna; Helicobia; natural enemies; parasitoid-host interaction.*

Um registro sem precedentes de parasitoidismo de Formicidae por uma mosca sarcófaga

Resumo: O registro do parasitoidismo de uma formiga Myrmecinae por uma mosca Sarcophaginae foi relatado no Cerrado brasileiro. A larva de um exemplar de *Helicobia* Coquillet (Sarcophagidae) se alimentou dos tecidos da cabeça de um soldado de *Atta laevigata* Smith, 1858 e um adulto macho emergiu de um pupário que se formou fora do hospedeiro.

Palavras-chave: *Savana Brasileira; Helicobia; inimigos naturais; interação parasitoide-hospedeiro.*

Introduction

Parasitoidism of ants by flies (Diptera) have been reported for single species of the families Tachinidae and Syrphidae and many species of the family Phoridae. The tachinid *Strongygaster globula* Meigen, 1824 (formerly called *Tamiclea globula*) has as its host young queens of the genus *Lasius* Fabricius, 1804 (Hymenoptera: Formicidae: Formicinae) (Schmid-Hempel 1998, Herting 2017), while the syrphid *Hypselosyrphus trigonus* Hull, 1937 is known as a parasitoid of workers of the tree ant *Neoponera villosa* (Fabricius, 1804) (Hymenoptera: Formicidae: Ponerinae) (Pérez- Lachaud et al. 2014). On the other hand, phorid flies are important parasitoids of a wide range of social insects, with ants as the main hosts (Disney 1994). Accordingly, interactions between phorid parasitoids and many members of the family Formicidae have been described, including species of the genus *Atta* Fabricius, 1805 and *Acromyrmex* Mayr, 1865 (Myrmicinae) of the tribe Attini (Disney 1994, Schmid-Hempel 1998, Bragança 2011, Uribe et al. 2014). Generally, phorids that attack ants are small flies that pursue workers along their trails or in foraging areas, ovipositing eggs on the heads or abdomens of the host (Erthal & Tonhasca 2000, Tonhasca et al. 2001, Bragança et al. 2002).

The large leaf-cutting ant *Atta laevigata* Smith, 1858, an eusocial insect widely distributed in Brazil, causes significant damage to agricultural and forest crops (Montoya-Lerma et al. 2012) and therefore, it is a target of biological control. We observed the interactions between phorid flies and leaf-cutting ants along their foraging trails to assess the levels of parasitoidism of *A. laevigata* in the Cerrado of the Central-West region of Brazil.

During fieldwork, we observed a single sarcophagid fly interacting with an *A. laevigata* soldier. The observation was noteworthy because sarcophagid species commonly establish predatory, kleptoparasitic, or parasitic interactions with butterflies, grasshoppers, moths, termites, wasps, and bees, but not with ants (Pape 1987, Schmid-Hempel 1998).

Material and Methods

Fieldwork was carried out in a Cerrado area (10°10′56.3″S, 48°18′23.8″W) close to the city of Palmas, Tocantins, Brazil. During the search for phorids attacking *A. laevigata* workers, an unidentified sarcophagid or tachinid was observed flying over, and sometimes walking on, the head and back of an individual soldier, who was moving unusually slowly manner.

The soldier was collected carefully using forceps, transferred to a small glass jar, and taken to the laboratory. However, the ant-attacking fly escaped during the capture attempt. Later that same day, the soldier was examined under a magnifying lens, and a larva was detected moving within the cephalic capsule. The ant was placed in a Petri dish, fed with 10% honey solution soaked into a cotton wool pad, and maintained at 26ºC in a rearing chamber. The larva developing inside the cephalic capsule was observed every 24 hours. The puparium was subsequently detached from the pad, transferred to a glass vial (100 mm high x 13 mm diameter), and maintained in the rearing chamber. An adult male fly emerged from the puparium (Figure 1A), and the specimen was identified by Dr. Cátia Mello Patiu (National Museum, Rio de Janeiro, RJ, Brazil), using a Sarcophagidae identification key for the genus (Pape & Dahlem 2010). Although the male holotype was fully described, the specimen, along with its description and undigitized drawings, was unfortunately lost in a fire that destroyed the Rio de Janeiro's National Museum on 2 September 2018 (personal communication, C. Mello-Patiu).

Results and Discussion

Approximately 72 hours after being placed in the rearing chamber, the soldier was confirmed dead, with the mandibles open. Further inspection revealed that the cephalic capsule was empty and the head devoid of tissues. Except for the mandibles, all other mouthparts were released from the cephalic capsule (Figure 2). The larva exited the capsule through the gap between the mandibles and formed a puparium between the cotton fibers inside the Petri dish. The fly emerged 12 days later and the fly was identified as a member of the genus *Helicobia* Coquillet, 1985 (Diptera: Sarcophagidae: Sarcophaginae) (Figure 1B).

Figure 2. Ventral face of the cephalic capsule of an Atta laevigata Smith, 1858 (Hymenoptera: Formicidae: Myrmicinae) soldier. Capsule width: 6.4 mm.

The association between Formicidae (*A. laevigata*) and Sarcophagidae (*Helicobia* sp.) is reported here for the first time. The biological characteristics of this interaction are comparable with those described for *A. laevigata* and the phorid *Apocephalus attophilus* Borgmeier, 1928, which is one of the several parasitoids of the family that oviposit on at least four other species of leaf-cutting ants (Erthal & Tonhasca 2000, Bragança & Medeiros 2006, Bragança 2011). In both interactions, the parasitoid larva develops inside the head of an *A. laevigata* worker, consumes the capsule contents, exits through the gap between the mandibles, and forms a puparium outside the host. When *A. laevigata* is parasitized by *A. attophilus*, the number of larvae per host ranges between 1 and 14, depending on the size of the cephalic capsule of the workers and soldiers, with the number of puparia also varying (Erthal & Tonhasca 2000, Bragança et al. 2016).

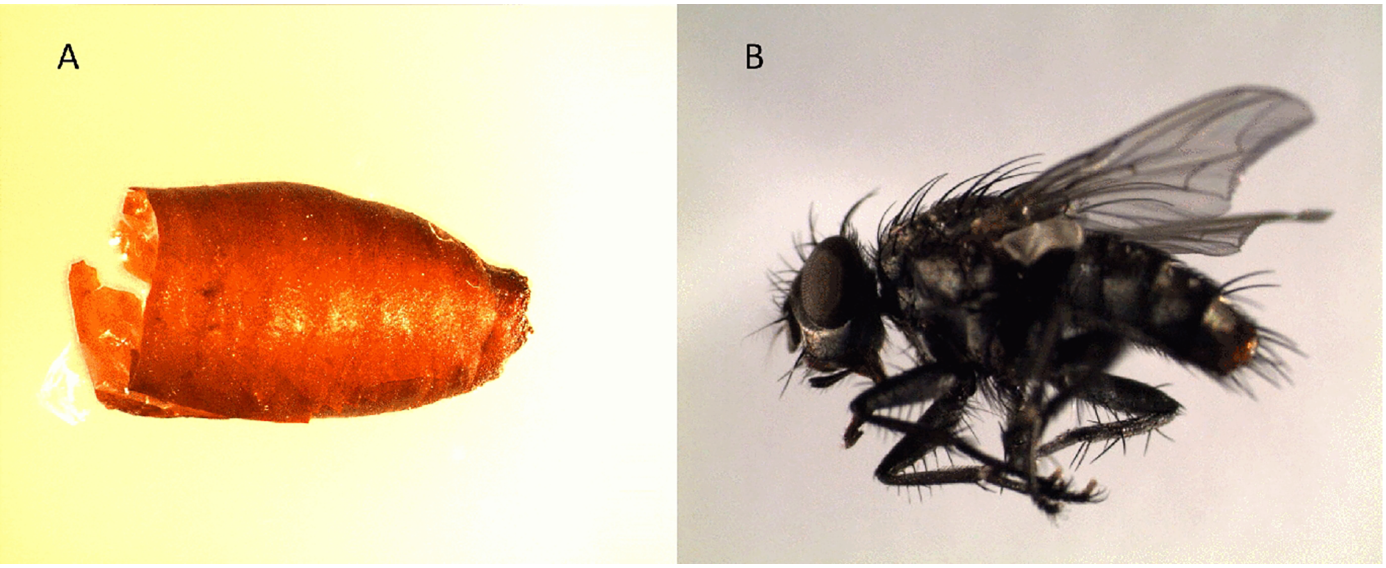

Figure 1. (A) Puparium of the *Helicobia* sp. Coquillet (Diptera: Sarcophagidae: Sarcophaginae) after emergence of the adult fly, showing the operculum detached from the rest of the puparium. Puparium length: 3.5 mm. (B) Adult male of the sarcophagid parasitoid.

However, in the novel interaction reported here, a single *Helicobia* sp. larva developed inside the head of an *A. laevigata* soldier, in which the cephalic capsule was 6.4 mm wide and the puparium was 3.5 mm in length, i.e., substantially larger than those of *A. attophilus* larvae (0.97 ± 0.11 mm [range from 0.86 to 1.23], n = 26; unpublished results). Interestingly, associations between *A. laevigata* and other phorid parasitoids, such as those of the genera *Eibesfeldtphora* Disney, 2009, and *Myrmosicarius* Borgmeier, 1928, almost invariably result in the development of a single larva per host. However, the puparium is generally formed between the mandibles of the ant, or inside its cephalic capsule or thorax (Bragança et al. 2002, Bragança et al. 2003, Bragança & Medeiros 2006).

The common biological characteristics of *A. attophilus* and *Helicobia* sp. differ from those of Tachinidae and Syrphidae flies that reportedly parasitize ants other than *A. laevigata*. For example, tachinid *S. globula* is an endoparasite that develops in the gaster of young queens (*i.e.*, colony-founding queens) of the black garden ant *Lasius niger* Linnaeus, 1758 or *Lasius alienus* Foerster, 1850. During the final instar, the larva exits the abdomen of the host through the anus and forms a puparium among the offspring of other ants, where it is raised by workers as if it were part of the colony itself (Gösswald 1950, Hölldobler & Wilson 1990). On the other hand, the larva of the syrphid *H. trigonus* consumes almost all contents of the *N. villosa* prepupa and forms its puparium inside the cocoon of the host (Pérez-Lachaud et al. 2014).

Regarding the behavioral attraction mechanisms, phorids seem to use long-distance olfactory cues to guide them to ant nests and foraging trails (Feener & Brown 1997). In the interaction between *A. attophilus* and *A. laevigata,* the female fly approaches its prey in the foraging area and walks among the workers aiming to insert its ovipositor into their mouths while they are cutting the leaves (Erthal & Tonhasca 2000). Other phorid parasitoids of *A. laevigata*, such as *Eibesfeldtphora tonhascai* Brown, 2001, and *Eibesfeldtphora erthali* Brown, 2001, have been observed to fly over workers and insert their ovipositors into the back of the head or gaster, respectively (Bragança et al. 2002).

No information is currently available regarding the mechanisms of attraction of sarcophagids to their hosts. During the field research reported herein, we observed that the putative sarcophagid fly performed short flights and touched the head of the soldier. The fly also walked incessantly over the back of the ant, sometimes touching the last portion of the abdomen. Since sarcophagids typically lay ready-to-hatch or newly hatched larvae (Pape 1987), the touching and walking activities of the fly could be described as larviposition behavior. However, later examination of the soldier revealed that there was a larva already developing inside its cephalic capsule. Hence, it is possible that the touching and walking activities of the fly represented a host-searching behavior and that the soldier was rejected because it was already parasitized. It is worth noting that discriminatory activities towards parasitized and non-parasitized ant hosts have been observed previously among phorid flies, albeit with low frequency (Bragança et al. 2009). Despite having no reports of *Helicobia* parasitoidism on Formicidae, there are parasitoidism records of this parasitic genus with other groups of the order Hymenoptera (e.g., bees) (Nowogrodzki & Morse 1990, Schmid-Hempel 1998).

Although the interaction between the leaf-cutting ant *A. laevigata* and the sarcophagid fly *Helicobia* sp. is atypical and rare, the form of parasitoidism resembles that observed between the same host and other flies, especially among phorids. Therefore, this sarcophagid species can be considered another natural enemy of leaf-cutting ants, which are destructive herbivores throughout South America.

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Author Contributions

Marcos Antonio Lima Bragança: funding acquisition, supervision, collection, writing.

Raquel Silva Acácio: collection and maintenance of the parasitoid fly.

Filipe Viegas de Arruda: writing and contribution to critical revision, adding intellectual content.

Marcos Antônio Pesquero: contribution to critical revision.

Conflicts of Interest

The authors declare no conflict of interest related to the publication of this manuscript.

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