

Notes on the sand fly fauna (Diptera: Psychodidae) in a region of Brazil

Notas sobre a fauna de flebotomíneos (Diptera: Psychodidae) em uma região do Brasil

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Abstract

The family Psychodidae has a cosmopolitan distribution with members that occur in many habitats, mainly in humid environments, and is most diverse in the tropics. Subfamilies Sycoracinae and Phlebotominae have females with hematophagous habits and the latter studied more due to medical and veterinary interest, since it includes species that can transmit diseases to animals and humans. The knowledge about the sand fly fauna in a region is extremely important for adequate monitoring and control measures for leishmaniasis. Thus, the objective of this study was to characterize Psychodidae fauna in relation to richness, abundance and molecular identification of *Leishmania* spp. in sand flies in southern Santa Catarina, Brazil. The collections were carried out between 2015 and 2016 in three cities in Santa Catarina, Brazil. Samples were taken near feeding places for domestic animals, urban forest and peridomicile areas. The insects were identified and female sand flies were submitted to molecular analysis to detect the presence of *Leishmania* spp.. A total of 4,200 insects were collected, 4,193 from the Sycoracinae subfamily and 7 Phlebotominae

from the *Nyssomyia neivai* and *Pintomyia fisheri* species. Of the studied municipalities, sandflies were registered in Tubarão and Imaruí and the most frequent habitat was the peridomicile areas. No samples were positive for *Leishmania* spp. In conclusion, the work highlights the presence of two species of sandflies, which were recorded for the first time in southern Santa Catarina.

Keywords: Psychodidae; Sycoracinae; Phlebotominae; Sand fly; Leishmaniasis.

Resumo

A família Psychodidae possui distribuição cosmopolita com membros que ocorrem em diversos habitats, principalmente em ambientes úmidos, sendo mais diversa nos trópicos. As subfamílias Sycoracinae e Phlebotominae apresentam fêmeas com hábitos hematófagos, sendo esta última estudada mais por interesse médico e veterinário, pois inclui espécies que podem transmitir doenças para animais e humanos. O conhecimento da fauna de flebotomíneos em uma região é de extrema importância para o adequado monitoramento e controle da leishmaniose. Assim, o objetivo deste estudo foi caracterizar a fauna de Psychodidae em relação à riqueza, abundância e identificação molecular de *Leishmania* spp. em flebotomíneos no sul de Santa Catarina, Brasil. As coletas foram feitas entre 2015 a 2016 em três cidades catarinenses. As amostras foram coletadas próximo a locais de alimentação de animais domésticos, floresta urbana e áreas de peridomicílio. Os insetos foram identificados e flebotomíneos fêmeas foram submetidos a análises moleculares para detecção de *Leishmania* spp. Foram coletados 4.200 insetos, sendo 4.193 espécimes da subfamília Sycoracinae e 7 Phlebotominae das espécies *Nyssomyia neivai* e *Pintomyia fisheri*. Dos municípios estudados, os flebotomíneos foram registrados em Tubarão e Imaruí e o habitat mais frequente foram as áreas de peridomicílio. Nenhuma amostra foi positiva para *Leishmania* spp. Em conclusão, o trabalho destaca a presença de duas espécies de flebotomíneos, que foram registradas pela primeira vez no sul de Santa Catarina.

Palavras-chave: Psychodidae; Sycoracinae; Phlebotominae; Flebotomíneo; Leishmanioses.

Resumen

La familia Psychodidae tiene una distribución cosmopolita con miembros que se encuentran en diferentes hábitats, principalmente en ambientes húmedos, siendo más diversos en los trópicos. Las subfamilias Sycoracinae y Phlebotominae tienen hembras con hábitos hematófagos, siendo esta última más estudiada por interés médico y veterinario, ya que incluye especies que pueden transmitir enfermedades a animales y humanos. El conocimiento de la fauna de flebotomos en una región es extremadamente importante para el seguimiento y control adecuados de la leishmaniasis. Así, el objetivo de este estudio fue caracterizar la fauna de Psychodidae en relación a la riqueza, abundancia e identificación molecular de *Leishmania* spp. en flebotomos en el sur de Santa Catarina, Brasil. Las recolecciones se realizaron entre 2015 y 2016 en tres ciudades de Santa Catarina. Las muestras se recolectaron cerca de sitios de alimentación de animales domésticos, bosques urbanos y áreas de peridomicilio. Se identificaron insectos y se sometieron a análisis moleculares hembras de flebotomos para detectar *Leishmania* spp. Se recolectaron un total de 4.200 insectos, 4.193 de la subfamilia Sycoracinae y 7 Phlebotominae de las especies *Nyssomyia neivai* y *Pintomyia fisheri*. De los municipios estudiados, se registraron flebotomos en Tubarão e Imaruí y el hábitat más frecuente fue el peridomicilio. Ninguna muestra fue positiva para *Leishmania* spp. En conclusión, el trabajo destaca la presencia de dos especies de flebotomos, que se registraron por primera vez en el sur de Santa Catarina.

Palabras clave: Psychodidae; Sycoracinae; Phlebotominae; Flebotomina; Leishmaniasis.

1. Introduction

The family Psychodidae comprises small Diptera that are characterized mainly by having a body covered with bristles (Sherlock, 2003). It has a cosmopolitan distribution with members that occur in many habitats, mainly in humid environments, and is most diverse in the tropics. The family has more than 3,000 described species subdivided into 6 subfamilies: Bruchomyiinae, Psychodinae, Trichomyiinae, Sycoracinae, Phlebotominae, and Horaiellinae (the last occurs only in East Asia) (Bejarano & Estrada, 2016; Pape et al., 2011).

Subfamilies Sycoracinae and Phlebotominae have females with hematophagous habits and the latter studied more due to medical and veterinary interest, since it includes species that can transmit different diseases (e.g., leishmaniasis) to animals and humans (Guimarães et al., 2014; Pape et al., 2011; Sherlock, 2003). According to Young & Duncan (1994) (Young & Duncan, 1994), the *Leishmania* spp. vectors in the Americas belong to *Lutzomyia*, but in a new classification by Galati (2003) (Galati, 2003) this genus was divided into many genera, especially *Lutzomyia*, *Migonemyia*, *Pintomyia*, *Bichronomyia*, *Psychodopygus*, *Nyssomyia* and *Trichophoromyia* (Galati, 2003; Young & Duncan, 1994). In addition, the largest number of species of sandflies is found in the Americas, with Brazil being the country with the greatest diversity of species (Galati, 2003).

Leishmaniasis is caused by an infection with *Leishmania* spp. and is among the most neglected diseases in the world, affecting poor populations, especially in developing countries. Leishmaniasis can occur in cutaneous and mucocutaneous (American Tegumentary Leishmaniasis) and visceral (Visceral Leishmaniasis) forms, depending on the parasitic species involved in the infection. Visceral leishmaniasis is the most worrisome form of the disease, and when not diagnosed and treated early, the mortality rate can reach 10% (Alves, 2009; de Brito et al., 2014; World Health Organization, 2010). According to the World Health Organization (WHO) (World Health Organization, 2010), almost two million new cases of leishmaniasis are reported world wide each year.

In Brazil, at 2015, 20.975 LTA cases (Ministério da Saúde, 2020a) and 3.558 LV cases were confirmed (Ministério da Saúde, 2020c). This country, which concentrates 95% of cases of VL in the Americas (Alvar et al., 2012), had the southern region considered free of the disease until 2009, but the number of cases has grown in recent years (Deboni et al., 2011). In Santa Catarina State, between 2011 and 2019, 14 LV cases were confirmed (Ministério da Saúde, 2020d) and, between 2010 and 2019, 193 LTA cases were confirmed (Ministério da Saúde, 2020b). At 2012 the State of Santa Catarina, reported the first autochthone cases of canine visceral leishmaniasis in state's capital (Figueiredo et al., 2012). Further, there are many problems with mapping the leishmaniasis cases as there are a large number of unreported cases (Gontijo & Melo, 2004). Thus, were reported revealed a new endemic area in the northeastern part of the state, with two *Leishmania* species present, whereas in the southern part the infection cases are predominantly imported, except for three *Leishmania (Leishmania) amazonensis* cases (Marlow et al., 2013).

The sand fly habitat in an area may be influenced by several factors, such as food and the rainfall index. With increasing deforestation and urbanization, these insects are also developing in peri-urban and urban areas, indicating their ability to inhabit anthropic environments (Feitosa & Castellón, 2006; Ramos et al., 2014; Souza et al., 2010). Thus, knowing the insect fauna is important so it can be compared with other regions to establish an epidemiological profile, since each region, depending on its location, temperature and other variables, may have different species (in quantity and variety).

Therefore, knowledge about the sand fly fauna in a region is extremely important for adequate monitoring and control measures for leishmaniasis. In addition, the sand fly fauna in Santa Catarina is poorly described, with few species listed for the state, and until now, no study had been carried out in the southern region. Thus, the objective of this study was to characterize Psychodidae fauna in relation to richness, abundance and molecular identification of *Leishmania* spp. in sand flies of the subfamily Phlebotominae in southern Santa Catarina, Brazil.

2. Material and Methods

2.1 Collection and identification

The study was cross-sectional with a quantitative approach and the collections were made out in three cities in Santa Catarina, Brazil: Criciúma (latitude 28°40'42" south and longitude 49°22'13" west), Tubarão (latitude 28°28'00" south and longitude 49°00'25" west) and Imaruí (latitude 28°20'42" south and longitude 48°48'59" west). In each city, there were three collection sites: (A) peridomicile area; (B) urban forest; and (C) near feeding places for domestic animals (chickens, pigs, dogs and cattle).

The geolocations of the different points are in Table 1. The insects were captured using CDC luminous traps from April 2015 to March 2016. The collections were made monthly for approximately 12 hours (~ 18:00 pm to 06:00 am) per sampling period. The collected insects were screened, separating, and those with a morphology similar to Psychodidae were further identified. Identifications were made using keys by Galati (2003) (Galati, 2003) and Sycoracinae was identified to subfamily. All insects selected were stored in 70% alcohol.

Table 1. Geolocation collection points.

Locality	Point A	Point B	Point C
Criciúma	-28.6973466, -49.3681427	-28.7207446, - 49.3697917	-28.7049809, - 49.3779168
Tubarão	-28.496528, -49.025939	-28.477459, -49.023666	-28.520065, -49.017660
Imaruí	-28.300931, -48.761454	-28.303216, -48.762705	-28.303346, -48.761259

Source: Authors.

2.2 Sand fly DNA extraction

DNA extraction was performed with a Gentra Puregene® QIAGEN tissue kit following the protocol modified by Quaresma *et al* (2011) (Quaresma et al., 2011): females were macerated in 100µl of cell lysis solution and 1µl of proteinase K was added to the mixture. The solution was homogenized by tube inversion and incubated at 55°C overnight. After incubation, 1µl of RNA se was added and homogenized by inversion and incubated for 30 minutes at 37°C. After this, the samples were incubated for 3 minutes on ice and 100µl of protein precipitation solution was added.

The tubes were vortexed for 20 seconds and then centrifuged for 5 minutes at 1,400 rpm. The supernatant was placed in a new tube, 300µl of isopropanol was added by inverting the remaining sample, the sequence another centrifugation at 1400 rpm for 5 minutes. The supernatant was discarded, and the tube was inverted and left to dry on absorbent paper. Subsequently, 70% alcohol was added to dry inverting it several times followed by centrifugation at 1400 rpm for 5 minutes. The supernatant was discarded, and the tube was inverted and left to dry on absorbent paper. Finally, 25µl of ultrapure H₂O was added for DNA hydration.

2.3 PCR identification

To determine the presence of parasites of the collected sand fly females, the extracted DNA samples were analyzed based on a PCR technique used for *Leishmania* ITS1 (Tail et al., 2000). For amplification, the pair of LITSR primers was used: 5'CTGGATCATTTTCCGATG 3' and L5.8S: 5'TGATACCACTTATCGCACTT 3'.

The reaction was prepared to a final volume of 25µl containing 5µl of the DNA sample, 2.5µl of 10x buffer solution, 0.75µl MgCl₂ (50mM), 0.5µl of 10mM dNTP mix, 1.25µl each of 10 µM primers (LITSR and L5.8S), 0.25µl of 10U/µl Taq DNA polymerase, 1.25 µl of dimethylsulfoxide and 12.25µl of sterile distilled water.

DNA amplification was conducted with an Eppendorf® Mastercycler Gradient automatic cycler using the following: initial denaturation for 2 minutes at 95°C, sequencing 35 denaturation replicates for 30 seconds at 95°C, annealing for 60 seconds at 53°C and extension for 60 seconds at 72°C. The final extension was at 72°C for 10 minutes.

Twenty nanograms of DNA extracted from *Leishmania braziliensis* (MHOM / BR / 75 / M2903) was used as the positive control and sterile distilled water was used as the negative control. The results were observed on 2.0% agarose gel stained with ethidium bromide and viewed under ultraviolet (UV) light.

2.4 Statistical analysis

The following data were analyzed: richness, abundance, sex, infected females, and seasons of the year. Data meeting the prerequisite for normality were subjected to an analysis of variance (ANOVA). Tukey's *post-hoc* test was used when significant differences were detected ($p < 0.05$). Non-parametric data were analyzed using the Kruskal-Wallis test by rank and median. A Mann-Whitney pairwise test was used when significant differences were detected ($p < 0.05$) (Sokal & Rohlf, 2011). The correlations between climatic variables (temperature and air humidity) and population density of Psychodidae were

evaluated by means of Spearman's coefficient ($p \leq 0.05$). All analyses were performed using the statistical software PAST v3.07 (Hammer et al., 2001).

3. Results

In our studies, from April 2015 to March 2016, it was possible to distinguish four seasons of the year, when 4,200 insects of family Psychodidae were collected, including two subfamilies: Sycoracinae and Phlebotominae. Subfamily Sycoracinae was represented by 4,193 specimens and subfamily Phlebotominae by 7 specimens of two species: *Nyssomyia neivai* (= *Lutzomyia neivai*) and *Pintomyia fischeri* (= *Lutzomyia fischeri*). The highest Psychodidae abundance was observed in Imaruí (57.2%), followed by Criciúma (30.6%) and Tubarão (12.2%). An analysis of Psychodidae abundance revealed a significant difference between the number of specimens collected in the different cities ($F = 27.1$; $p < 0.05$). Psychodidae abundance was significantly different between the three collection sites, considering the three cities ($F = 7.2$; $p < 0.05$), with the highest abundance near feeding places for domestic animals (53.5%). Subfamily Phlebotominae was registered in Imaruí and Tubarão (Table 2).

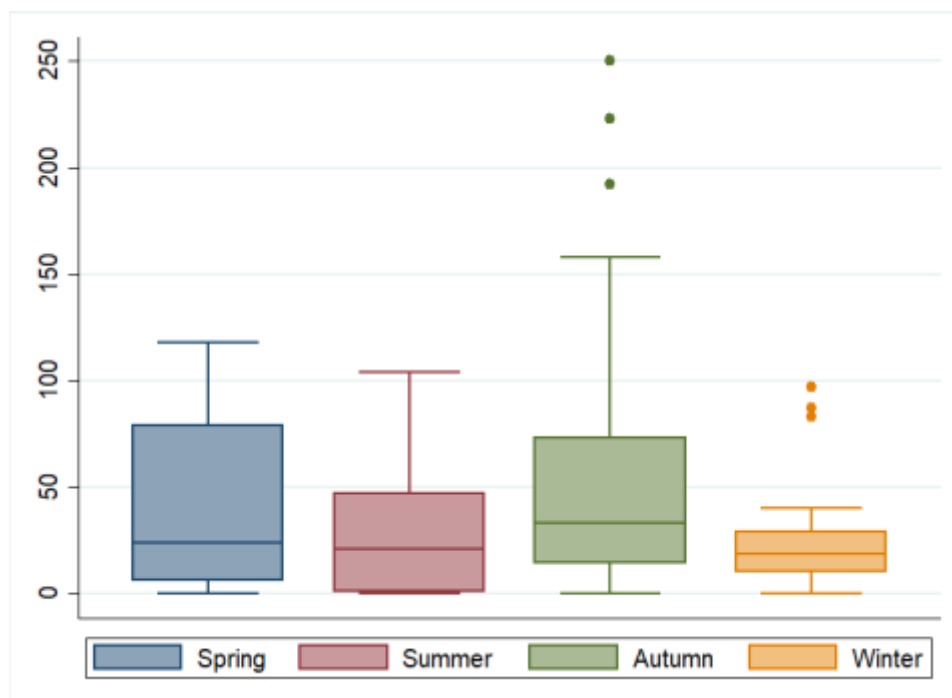
Table 2. Quantity of specimens of the family Psychodidae and subfamily Phlebotominae collected from April 2015 to March 2016, including sites and cities where the collections were made.

City	Collection Sites	Family Psychodidae	Subfamily Phlebotominae	
		Abundance	Abundance / Richness	
			<i>Nyssomyia neivai</i>	<i>Pintomyia fischeri</i>
Criciúma	Urban Forest	406	-	-
	Animal Food Source	809	-	-
	Peridomicile	70	-	-
Imarui	Urban Forest	667	-	-
	Animal Food Source	1.109	-	-
	Peridomicile	627	2♂ 2♀	1♀
Tubarão	Urban Forest	157	1♂ 1♀	-
	Animal Food Source	329	-	-
	Peridomicile	26	-	-

Source: Authors.

There was no significant difference in abundance between the different months, considering the three cities ($H = 9.5$; $p > 0.05$). In order to find other factors relevant to the presence of phlebotomine in the cities, we categorized the seasons of the year and observed that the family Psychodidae was constantly reported in the collections; the data showed a greater number in autumn and lower number in winter (Figure 1).

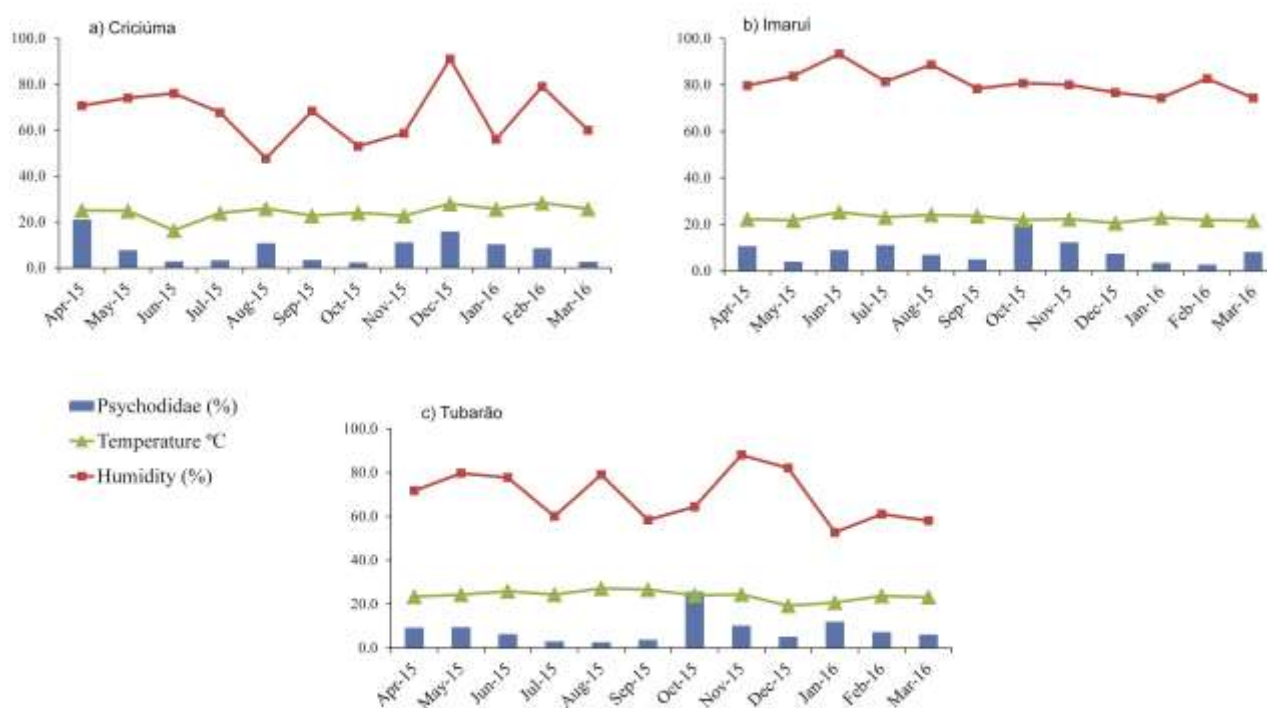
Figure 1. Quantity of Psychodidae specimens in relation to the four seasons of the year.



Source: Authors.

For sand fly, there were no specimens in winter. There was a significant difference in Psychodidae abundance between the seasons of the year ($F = 4.4$; $p < 0.05$). The correlation between the number of Psychodidae captured and the climatic variables (temperature and relative air humidity), in each city, is shown in Figure 2. The climatic variables evaluated showed a positive Spearman correlation coefficient in relation to Psychodidae density, except for temperature in Tubarão. However, without significant differences (Figure 2).

Figure 2. Correlation between population density of Psychodidae and climatic variables (temperature and relative humidity of the air) in three cities in Santa Catarina State, Brazil, from April 2015 to March 2016. a) Criciúma; Psychodidae vs. temperature (Spearman's rho = 0.34, n = 12, P = 0.27); Psychodidae vs. humidity (Spearman's rho = 0.18, n = 12, P = 0.58). b) Imaruí; Psychodidae vs. temperature (Spearman's rho = 0.06, n = 12, P = 0.85); Psychodidae vs. humidity (Spearman's rho = 0.04, n = 12, P = 0.89). c) Tubarão; Psychodidae vs. temperature (Spearman's rho = -0.37, n = 12, P = 0.23); Psychodidae vs. humidity (Spearman's rho = 0.04, n = 12, P = 0.89).



Source: Authors.

Finally, to verify the possible infection by *Leishmania* spp. in the sand fly females collected, molecular analysis techniques were used, which showed that all samples were negative for *Leishmania* spp. The results of the PCR technique used to investigate *Leishmania* infection in the sand flies collected were not positive for any of the analyzed samples.

4. Discussion

This study collected two subfamilies of Psychodidae: Sycoracinae and Phlebotominae. In a similar study in the city of Florianópolis, Santa Catarina State, 413 sand fly 658 were collected distributed in several species: *Lutzomyia fischeri*, *Lu. Migonei*, *Lu. Neivai*, *Lu. edwardsi*, *Lu. tupyinambai* and *Lu. firmatoi*. Although the author did not provide details of the collection point characteristics, it can be inferred that the number of *Lu. neivai* specimens collected was close to that obtained in our study because the author reported 10 females of this species; however, for *Lu. fischeri* the author reported 39 females (Dias et al., 2011).

In another study in Santa Catarina, in the city of Piçarras, Guimarães *et al.* (2014) (Guimarães et al., 2014) observed three species: *Lu. neivai*, *Lu. fischeri* and *Lu. aroyzay*. When comparing the similarity between the collection points and the

number of individuals collected, these authors collected 4 *Lu neivai* in peridomicile area, while in the present study 2 males and 2 females were collected in a peridomicile area in Imaruí, as well as 1 male and 1 female in an urban forest in Tubarão. Beyond this point, the authors also observed a greater predominance of this species near residences. For *Lu. fischeri*, the authors reported the same number of individuals that were found in this study; for both studies, the presence of 1 female was observed for point near banana plants (Guimarães et al., 2014).

As found in the present study, for collections made in eastern Santa Catarina in 2000 (Marcondes et al., 2005), and in a study conducted in the northeast of state at 2008 (Grott et al., 2014), was observed *Nyssomyia neivai* as a predominant phlebotomine species. Similar results were also obtained in a study conducted during 2012 in Paraná State (dos Santos et al., 2016). This species of phlebotomine is often found in peridomiciliary regions (dos Santos et al., 2016; Grott et al., 2014; Marcondes et al., 2005).

Pintomyia fischeri has been recorded in the states of the South Region of Brazil and is essentially a wild species, although it has already been found in peridomestic rural habitats (Pita-Pereira et al., 2011). Although only one specimen of this species was collected in the present work, the habitat characteristics that Pita-Pereira *et al.* (2011) (Pita-Pereira et al., 2011) correlate with encountering *P. fischeri* correspond to the similarity of the collection point where the species was found. Considering the abundance of *P. fischeri* in deforested areas, it can be suggested that this species is well suited to live in the surroundings of forests and environments with anthropic changes (Pita-Pereira et al., 2011).

The geographic distribution of Psychodidae species can be influenced by physical barriers, rainfall, vegetation, luminosity and abundance of vertebrate hosts (Aguilar et al., 1985). It is also known that the presence of animals influences the density of sand flies near human dwellings and, consequently, increases the transmission risk of *Leishmania* species to humans (Arias et al., 1985; Lainson et al., 1983; Rangel & Lainson, 2009).

Individuals of family Psychodidae were collected in peridomicile, urban forest and animal feeding source areas; a greater quantity was collected in the city of Imaruí, followed by the cities of Criciúma and Tubarão. For subfamily Phlebotominae the records were from the cities of Imaruí and Tubarão. The greater number of individuals for both Psychodidae and Phlebotominae in Imaruí is probably related to the fact that this city has a large amount of vegetation, even in places near the peridomicile area. In Tubarão, the sand fly specimens were found in a region near urban forest, thus corroborating authors who noted that the majority of phlebotomine species are essentially wild, although they have been found in rural peridomestic habitats (Feitosa & Castellón, 2006; Guimarães et al., 2014; Marlow et al., 2013).

When the collection points were compared, we found a predominance of individuals close to the animal food source, possibly due to the diversity of ecotypes that comprise the sites selected as an animal food source and to the eclectic habits of the captured species, since in these areas there are domestic animals such as dogs, birds, pigs and cattle. On the other hand, the peridomiciliary environment has more human interference, which probably hinders the adaptation of some species of sand flies (Rêgo et al., 2014; Ximenes et al., 1999). It is important to mention that the peridomicile areas of the houses studied have mostly rural characteristics with the presence of domestic animals and plantations of fruit trees and grains that provide an environment with refuges and food for sand flies and the organic matter necessary for the development of immature forms of these insects. This may explain the occurrence of sand fly records in these areas, even in low numbers.

In Paraná State, researchers evaluated the seasonality of phlebotomines and demonstrated that in hot and humid seasons of the year these insects are captured in greater numbers (Teodoro et al., 1993, 2001). The fact that southern Santa Catarina State has few sand flies may explain the low number of leishmaniasis cases, since this disease is not endemic to this region, although we know that there is a serious problem of unreported cases (Gontijo & Melo, 2004). Thus, constant monitoring of

circulating species is important since, when an increase of species or specimens is recorded over years, control methods must be considered that can prevent this disease from becoming endemic to southern Santa Catarina.

All sand fly samples that were subjected to molecular analysis had negative results, which is probably related to the low number of samples. Another factor that contributes to the lack of protozoan detection is that in non-endemic areas the circulation of this protozoan is low. Dias *et al.* (2010) (Dias *et al.*, 2011) evaluated the natural infection of sand flies by *Leishmania* spp. in the city of Florianópolis, using the SSUrRNA PCR technique, and obtained 658 specimens of sand flies of the following species: *Lutzomyia fischeri*, *L. migonei*, *L. edwardsi*, *L. tupynambai*, *L. firmatoi* and *L. neivai*. However, of the 136 "pools" per species of sand fly captured, only 9 pools were positive for *Leishmania* spp..

Therefore, should be taken into account that the epidemiology of leishmaniasis is conditioned the presence of vectors (phlebotomines) that can transmit the etiological agent *Leishmania* spp. and the presence of wild and synanthropic mammalian hosts and/or reservoirs (Souza *et al.*, 2010). The great diversity of vectors, reservoirs and agents influence changes in the epidemiological patterns of leishmaniasis, and the influence of humans on the environment also makes it difficult to control the disease (de Brito *et al.*, 2014).

5. Conclusion

Clinical, biological and epidemiological characterization studies are fundamental to the development of individualized action and prevention strategies, since transmission patterns, vector species, leishmaniasis species, and response to treatment can be very different in some regions. Thus, it is important that we better understand which species are circulating in our study area. The present study updated and expanded the knowledge of the geographic distribution of leishmaniasis vectors, which may assist both in the acquisition of knowledge about the behavior of species, as well as in the strategies of surveillance and control of this disease. The areas without confirmed records, but suitable for the occurrence of the species, should be verified in future studies of survey of phlebotomine fauna. We suggest that the monitoring of these species to be accomplished out periodically in other municipalities in the south of Santa Catarina in addition to those highlighted in this study so that you can have an overview of the recurrent species and make a comparison with those that have already been listed for the state.

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