Seasonality of Sand Flies (Diptera: Psychodidae) in Cave Microhabitats and The Rediscovery of Sergentomyia Minuta (Rondani, 1843) After Fifty Years

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Research Article

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Abstract

Background: In a countrywide study with the aim to update the knowledge on diversity of sand fly species in Romania, a sand fly population was observed in an isolated system of cave microhabitats. The caves are located in the protected area of Canaraua Fetii, Dobrogea region, southeastern Romania. The highest sand fly diversity was recorded at the above-mentioned area between 1968 and 1970. This work presents an abundance study, which was conducted in order to estimate the seasonal variation of the sand fly species in correlation with the particular environmental factors of the isolated system of cave microhabitats.

Methods: Sand flies were collected between May and October 2020 from one trapping site of interest, from Canaraua Fetii. The trapping site consisted of a system of cave entrances. CDC miniature light traps, and sticky traps were used to collect insects from the exterior walls of the cave entrances. Species identification of collected sand flies was done using morphological keys. Statistical analysis of the trapping and climatic data was performed.

Results: From all collected sand flies, 99.63% (818/821) were *Phlebotomus neglectus*, 0.12% (1/821) *Ph. balcanicus*, and 0.25% (2/821) *Sergentomyia minuta*. Sand fly activity was firstly observed in 2^{nd} of July and lastly in 24^{th} of September. A monomodal abundance trend was present, with the peak activity between 16^{th} and 17^{th} of July. The analysis of the climatic data showed correlations between the total number of captured sand flies and both, average temperature and average relative humidity. The total number of collected specimens was statistically higher when CDC miniature light traps were used compared to sticky traps. The number of females on the sticky traps was significantly higher than the number of males on the same trap type. When compared with the sticky traps, a significantly higher number of males were collected by CDC miniature light traps. This is the first record of *Se. minuta* in Romania after 50 years of no records (despite the trapping effort of the last five years in the country). Also, *Ph. sergenti*, previously present in this location, was not found.

Conclusions: In the investigated natural habitat, the diversity of the sand fly species appears to have changed, with the predominance of *Ph. neglectus* instead of *Ph. balcanicus* and *Se. minuta* (recorded as the two predominant species in 1968-1970). A monomodal abundance trend was observed as in other regions of the country. The sand fly activity in this particular cave microhabitat appears to be longer than in other regions in Romania. A longer sand fly activity increases the zoonotic risk of various pathogenic species' transmission, with an impact in the public health, as sand flies are important insect vectors.

Background

In both the Old and the New World, sand flies (Diptera, Psychodidae, Phlebotominae) of the genus *Phlebotomus* and *Lutzomyia* are important hematophagous insects of public health and veterinary concern [1]. The major vectorial role in the transmission of parasites of genus *Leishmania*

(Kinetoplastida, Trypanosomatidae) gave sand flies an important status among other vector insects. Their vectorial role was also confirmed for other bacterial and viral pathogens [2].

In southern Europe, and more particularly in the Mediterranean basin, which is highly endemic for zoonotic visceral leishmaniasis in humans (VL), and canine leishmaniasis in dogs (CanL), both caused by *Leishmania infantum*, sand fly species are abundantly present [2]. In Romania, three sand fly species are present that are confirmed vectors for *L. infantum*: *Phlebotomus perfiliewi*, *Ph. neglectus*, and *Ph. balcanicus* [2, 3, 4]. The important vectorial role of sand flies and the permanent risk of new foci of disease emergence requires a permanent surveillance in both vector presence, abundance and disease epidemiology, mainly at the limit of their distribution [5].

In Romania, eight sand fly species were recorded between 1910 and 1970, namely *Phlebotomus perfiliewi, Ph. neglectus, Ph. balcanicus, Ph. papatasi*, *Ph. alexandri*, *Ph. sergenti, Ph. longiductus*, and *Sergentomyia minuta* [6]. The highest sand fly diversity recorded in the country between 1968 and 1970 was found in the protected natural habitat of Canaraua Fetii, Dobrogea region, southeastern Romania, with four sand fly species: *Ph. neglectus*, *Ph. balcanicus*, *Ph. sergenti*, and *Se. minuta* [7]. In more recent studies conducted between 2013 and 2018, only five sand fly species were identified in Romania: *Ph. perfiliewi*, *Ph. neglectus*, *Ph. balcanicus*, *Ph. papatasi*, and *Ph. sergenti* [3, 4]. In the present, Mehedinţi Plateau (southwestern Romania) appears to have the highest sand fly species diversity in Romania, with five species recorded [3]. Additionally, one more sand fly species of subgenus *Transphlebotomus* was reported in Canaraua Fetii [8]. Up to date, there is one single study reporting the seasonality of sand flies in Romania [4] which revealed a monomodal type of abundance trend for *Ph. perfiliewi*, with a single activity peak at the beginning of August, in the northeastern region of the country.

The present study was designed to re-evaluate the sand fly diversity and abundance in the protected natural area of Canaraua Fetii, a sylvatic area with a history of high sand fly diversity and to describe the seasonal trends of sand flies in a cave microhabitat.

Materials And Methods

Study area and design

The study was conducted in the protected area of Canaraua Fetii in southeastern Romania (44.07302N, 27.64289E), where a population of sand flies has been observed during previous field studies [7, 8]. The protected area is situated in the southwestern part of Dobrogea Plateau. It is a limestone canyon, carved by a former river among hills forming a plateau. It has deciduous forests on both sides and typical short-grass steppes on top. The valley is moist (a temporary brook crosses, with slow-flowing water following rains), while the plateau is drier. Elevation is 100-130 m on plateau, and 18-26 m in the valley. A high diversity of animal species, with important bird and bat populations are present [9].

Between 21st of May and 8th of October 2020, insect collections were made by the use of two CDC miniature light traps set 12 times, for two consecutive nights on the exterior walls of the cave entrance.

The light-attraction collections were complemented by the use of the sticky traps as these were demonstrated to influence the proportion of males and females collected [10, 11]. Sticky traps consisted of A5 format white paper (148 mm × 210 mm) coated with castor oil; a fixed number of sticky traps per site (n = 20) was set in each trapping site during the sampling period. All traps were set overnight (19:00h-05:30h) on the very same place for the entire study, close to the walls, at a 1.5 m height from the ground (Figure 1).

The frequency of insect collection was initially performed at a two-week interval, until the first positive collection. After that, the insect collection was weekly performed. After the last sampling in August, the collections were again at two weeks in September, and finally stopped after two consecutive negative samplings. Overall, 12 trapping periods were done (Table 1).

The total number of light traps placed in the study site was 48 (2 CDC miniature light traps × 1 site × 2 consecutive nights × 12 sampling times. A total surface of 14.4 m^2 [0.03 m^2 (standard for A5) × 20 sticky traps × 2 (both sides of the paper) × 12 sampling times] of sticky traps immersed in castor oil was used.

Species identification

After each trapping night, insects were collected, stored in 70% ethanol and transferred to the laboratory for species identification. Sand flies were separated from other insects. The heads and genitalia of each specimen were dissected and individually slide-mounted. The slide-mounting was done in Swan solution (chloral hydrate/acetic acid/Arabic gum). Entomological keys [12, 13, 14] were used for species identification. The morphological identification of the species was based on specific features of the pharynx and external genitalia in males, and pharynx and internal genitalia in females.

Environmental data collection

The minimum, maximum, average daily temperatures and average relative humidity for the study locality were collected from the Romanian National Meteorological Administration (RNMA) for each day of the sampling period (see Supplementary material 1).

Data analysis

Shapiro-Wilk normality test was used to assess the distribution of data. The impact of environmental factors (average, minimum and maximum temperature, and relative humidity) on sand fly population dynamics were evaluated using Spearman's correlation. The strength of correlation was assessed based on the Spearman correlation coefficient (r_s) (0.00–0.19: very weak; 0.20–0.39: weak; 0.40–0.59: moderate; 0.60–0.79: strong; 0.80–1.0: very strong). Chi-square goodness of fit, Pearson's chi-squared and nonparametric Mann–Whitney U tests were used to compare the number of collected sand flies by trap type, gender and sampling periods. Linear regression analyses were performed in order to establish the existence of a linear relationship between the number of collected sand flies and environmental factors. Prediction models for the presence or absence of phlebotomine sand flies, based on humidity and average temperatures, were performed using logistic regression. *P* values less than 0.05 were considered statistically significant. Data were analysed using R software v. 4.0.5.

Results

From all (n = 821) collected sand flies, 99.63% (818/821) were *Ph. neglectus*, 0.12% (1/821) *Ph. balcanicus*, and 0.25% (2/821) *Se. minuta* (Table 1), identified by morphological characters (Fig. 2). As *Se. minuta* was trapped after a period of 50 years without records, an updated map of distribution was provided (Fig. 3). From the total number of collected individuals, the number of females (57.50%; 472/821) was statistically higher than the number of males (42.50; 350/821), at a p value < .001 (Table 1). From the total number of females, 3.60% (17/472) were engorged. No gravid females were observed.

Nine trapping periods out of twelve were positive for sand fly collection, between 2nd of July until 24th of September (Table 1). The highest abundance was recorded at the middle of July (16th -17th) (4th trapping period), accounting for 35.20% (289/821) of all captured sand flies (Fig. 4) resulting in a single activity peak (monomodal abundance trend). The total number of collected sand flies was statistically higher in the 4th, 5th, and 6th trapping period when compared to the rest of the trapping periods (p < .001) (Table 1). Significantly more males were collected in the 3rd trapping period than in the 4th one and the rest of the trapping periods (p < .001). Blood-fed females were caught in trapping periods 4–10 (Table 1), with no statistical difference between trapping periods.

The statistical analysis of the climatic data showed a moderate correlation between the number of captured sand flies and the average temperature ($r_s = 0.56$, $p = .004$). A strong negative correlation was also identified between the number of collected phlebotomine sand flies and the average relative humidity values of the collection day ($r_s = -0.63$, $p < .001$) as well as for the ones of the last 5 days, prior the collection day ($r_s = -0.58$, $p = .003$). In two logistic models that were created, associations between the presence/absence of sand flies in each trapping period, the corresponding average temperature (Fig. 5, p = .048) and average humidity values (Fig. 6, p = .038) were identified.

Regarding the trapping type, the total number of collected specimens was statistically higher when CDC miniature light traps were used compared to sticky traps, at a p value < .001. Among the total number of collected sand flies by sticky traps, the number of females (70.37%, n = 114) was statistically higher than the number of males (29.63%, n = 48, p < .001). Moreover, a significantly higher number of males were collected by CDC miniature light traps than by sticky traps (p = .01).

Discussion

This study presents the first detailed analysis of the seasonal activity of *Ph. neglectus* in Romania, apparently the most abundant species in the country [3]. This is the highest number of trapped individuals of *Ph. neglectus* to date in Romania [3, 4, 6, 7]. The species belongs to the *Ph. major* complex which currently comprises five other species, widely distributed in the Old World. *Phlebotomus neglectus* is the only species of the complex present in the south and southeastern regions of Europe [15]. In Romania, the species was historically reported in southwestern, southern and southeastern regions of Romania, along the Danube Valley and Bărăgan Plain [7, 16]. In other recent field studies, its presence was limited to southwestern part of Romania, in Mehedinţi Plateau, where it was present mostly peridomestically, outdoor [3].

The present study was conducted in the nature reserve of Canaraua Fetii, previously investigated for sand fly presence, between 1968 and 1970 [7]. At that time, four sand fly species were present: *Ph. balcanicus*, and *Se. minuta* (the two most abundant species), also *Ph. neglectus*, and *Ph. sergenti*. In the present study, the diversity and abundance of the sand fly species appears to have changed in this location, with *Ph. neglectus* the most abundant species (99.63%), and a very low abundance of *Ph. balcanicus* (0.12%), and *Se. minuta* (0.25%). *Phlebotomus sergenti* was not present at the trapping period of the current study.

The peak activity of *Ph. neglectus* (mid-July) was earlier with approx. 15 days when compared to another evaluated species in Romania, *Ph. perfiliewi* (monomodal abundance trend with the peak activity at the beginning of August) (Fig. 7) [4]. The earlier and longer activity frame of *Ph. neglectus* (southeastern Romania), compared with the one of *Ph. perfiliewi* (northeastern Romania) might be due to geographical position, microhabitat, and climatic differences (statistically supported in both studies) [4]. In another recent study of *Ph. mascittii*, the predominant sand fly species in Central Europe, a monomodal abundance trend was also present in Austria, with variations of the peak activity to July and August [17]. Located at a similar latitude as Romania, the sand fly activity in Austria started earlier, by late June, and ended earlier, by the end of August [17]. A monomodal abundance trend was also observed for other sand fly species, such as *Ph. ariasi* in France, *Ph. kandelaki*, and *Ph. balcanicus*, in Georgia [18].

Considering the Balkans region, a recent review revealed that *Ph. neglectus* was also the most abundant species in 2014 and 2016 in Serbia, Kosovo, Bosnia and Herzegovina, Montenegro, Croatia, Slovenia, and the second most abundant species in Bulgaria, and North Macedonia [19]. The abundance trend for *Ph. neglectus* can also be bimodal (two peaks of activity), a main characteristic when the species is located in the warmer, Mediterranean climate (e.g., Greece or Cyprus) [18]. For other sand fly species, up to three density peaks and substantially longer activity periods were observed in countries with lower latitudes, such as Portugal or Turkey [18].

Sergentomyia minuta was the second most abundant sand fly species present between 1968 and 1970 in Canaraua Fetii [7]. In the present study conducted after 50 years in the same location, only two specimens of *Se. minuta* (one male and one female) were trapped. Between 2013 and 2018, the species was not trapped in other sampling sites in Romania, despite the trapping effort [3, 4]. A possible explanation for the apparent lacking in the past years could be the trap type and the sampling date and effort. In the historical data [7], only sticky traps were used to collect sand flies, while in recent studies conducted in Romania between 2013 and 2018, only CDC miniature light traps were used [3, 4]. The two *Se. minuta* specimens (Table 1) were trapped with the sticky traps, while none were trapped with the CDC miniature light traps. Regarding the sampling date, it appears that *Se. minuta* was active and present at the beginning of July, while most of the trapping effort conducted in Romania so far was focused on the end of July-beginning of August [3]. The chosen trapping period was based on several available data: another seasonality study conducted in Romania, of *Ph. perfiliewi*, was assessed and the results shown the peak activity at the beginning of August [4]; the same species, *Ph. perfiliewi*, was active only after the average minimum temperature for the previous 7 days was above 15°C [4]; in another study, under laboratory conditions, another species, *Ph. papatasi* (recorded also in Romania) showed no larval and pupal development at 15°C and a mean temperature of at least 18°C was necessary for successful rearing [20]. In a more recent study, the earliest activity of *Ph. mascittii* was noticed after 5 consecutive days of mean temperature values above 15°C and minimum temperature values above 10°C [17].

In another study from eight Balkan countries (some of them neighbouring Romania), *Se. minuta* appeared to be one of the less abundant sand fly species in Bulgaria, North Macedonia, Serbia, Kosovo, Bosnia and Herzegovina, and Montenegro. In Slovenia, the species was not present in the evaluated trapping sites. On the other hand, in Croatia the species appeared to be the second more abundant species [19].

The third sand fly species present in the current study, *Ph. balcanicus* was one of the two most abundant species in Canaraua Fetii between 1968 and 1970 [7]. *Phlebotomus balcanicus* was also identified in North Macedonia, Serbia, Kosovo, Montenegro [19], Turkey, Armenia and Georgia [18].

The changes in the sand fly composition in Canaraua Fetii might have been influenced by a series of factors, such as: (i) demographic and human behavioural changes; (ii) changes in the host species availability; (iii) ecological changes; (iv) climatic changes; (v) use of insecticides. The selected study area (Canaraua Fetii Natural Reserve) has had a sinuous path in becoming a protected area. In the past, a stone quarry with employees was running. Human activity was present and with that, the availability of domestic hosts. The domestic animal host availability has changed and transitioned to a wildlife host after the stone quarry close [21]. This might have had influenced the changes in the sand fly fauna. Nowadays, even if the site is a protected area, a monastery was recently opened in 2012, and the tourist pilgrimage started. As a protected natural area, the abundance of animal species attracts an important number of outdoor enthusiasts. As for the insecticide use, DDT was used in Romania between 1958 and 1964 [6]. There are no data that indicates aerial spraying over this location, but since the previous sand fly sampling (1968–1970) in the area which was done after the DDT campaign, we eventually consider that this is less likely to be the cause of sand fly species composition change.

Conclusions

This is the first study conducted in Romania to evaluate the seasonal activity of *Ph. neglectus*, one of the vector species of *L. infantum*, in a six-month interval. In the reinvestigated natural habitat, the diversity of the sand fly species appears to have changed, with the predominance of *Ph. neglectus* instead of *Ph. balcanicus* and *Se. minuta.* The sand fly activity in this particular cave microhabitat appears to be longer than in other regions in Romania (July-September). A longer sand fly activity increases the disease transmission risk, to both humans and animals. The peak density of *Ph. neglectus* was observed in the middle of July, providing scientific data which can be further used during awareness campaigns for veterinarians and public health professionals. More seasonality studies are necessary to establish if the present activity pattern of *Ph. neglectus* might have variations in other geographical areas, as the present data could not be generalised to the entire Romanian territory.

Abbreviations

VL: visceral leishmaniasis (in humans); CanL: canine leishmaniasis;

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CDC and ADM designed the study. CH, DP, MM, and VA participated in the field work. CDC carried out the morphological speciation of sand flies. LCP carried out the statistical analysis of the study. CDC performed the GIS data management and drafted the original manuscript. ADM, CH, LCP, DP, MM, and VA critically revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Figure 1

The trapping site in Canaraua Fetii, Dobrogea Region, Romania

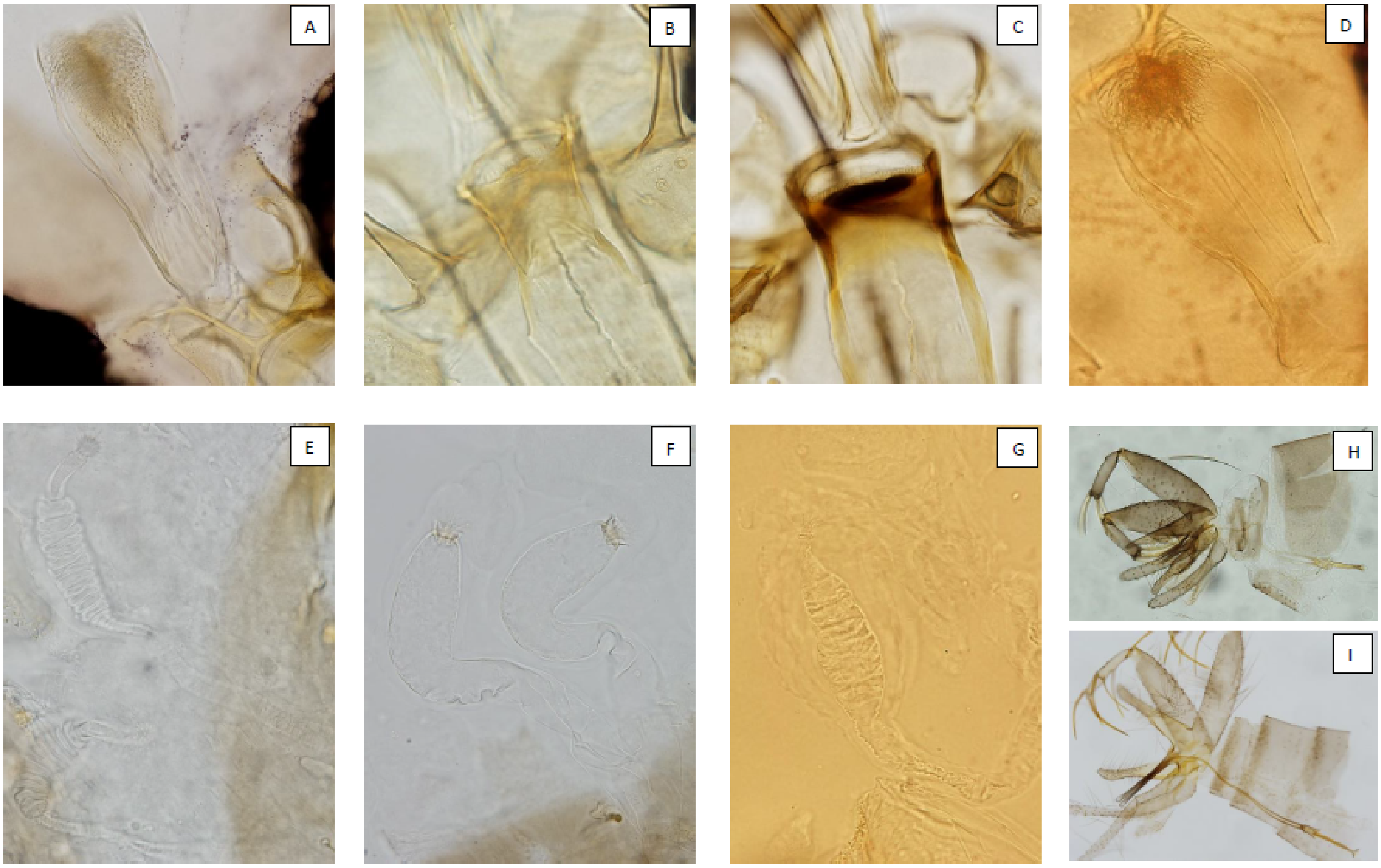

Figure 2

Morphological details for the three sand fly species: A - pharynx of Ph. neglectus; B - cibarium of male Se. minuta; C - cibarium of female Se. minuta; D - pharynx of Ph. balcanicus; E - spermathecae of Ph. neglectus; F - spermathecae of Se. minuta; G - spermathecae of Ph. balcanicus; H - aedeagus Se. minuta; I - aedeagus Ph. neglectus

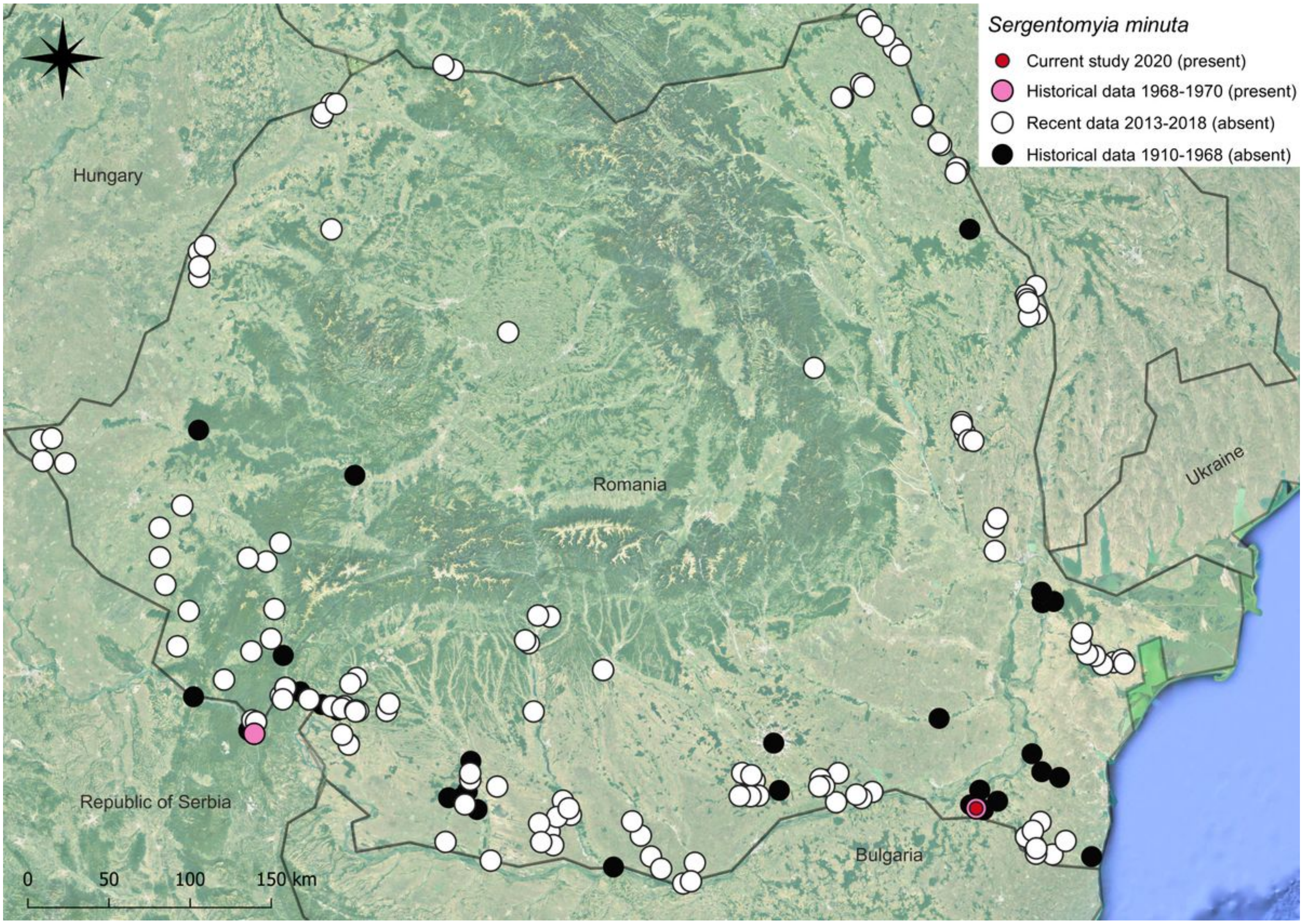

Figure 3

Distribution of Se. minuta in Romania

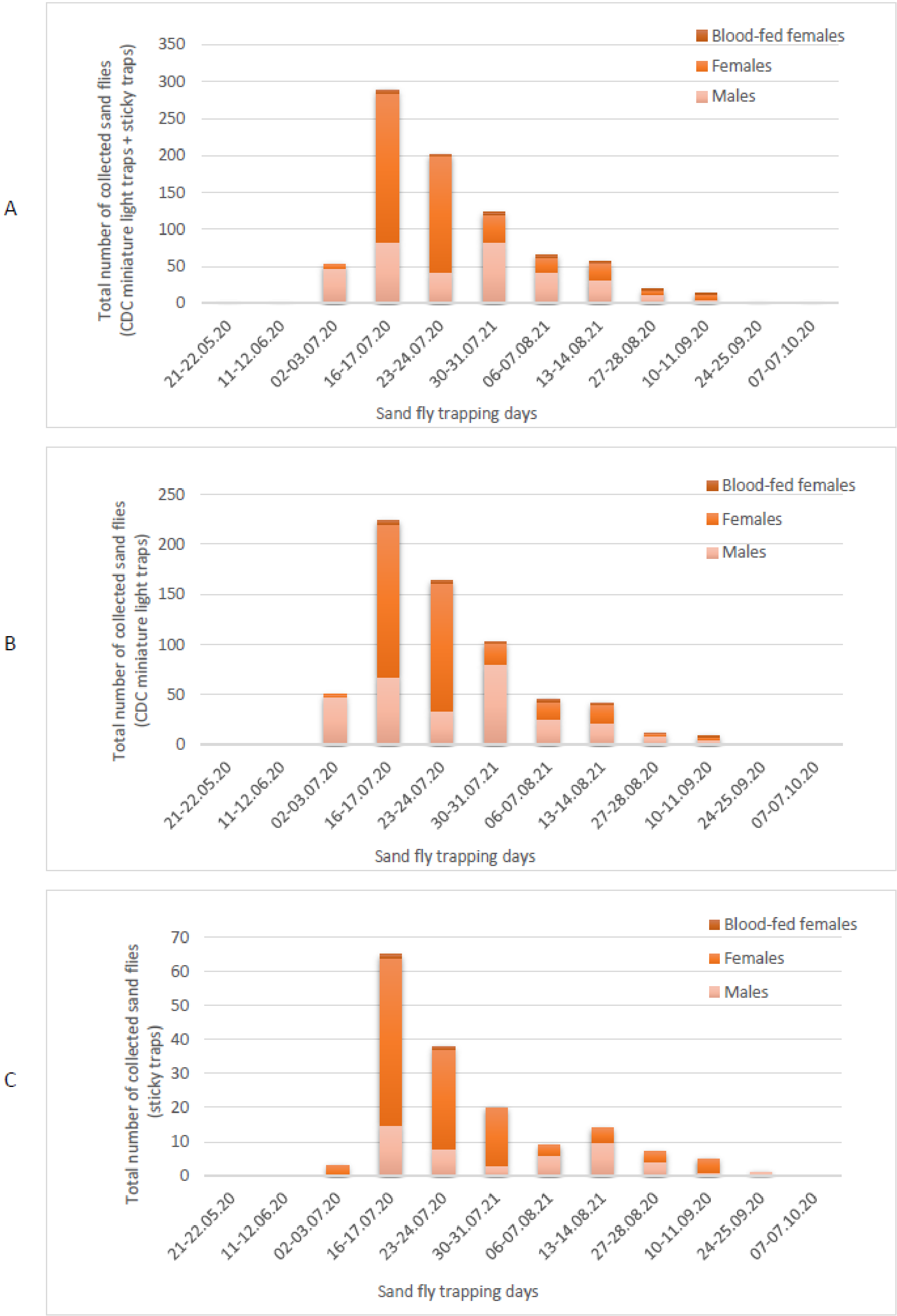

Figure 4

Seasonal abundance of sand flies in Canaraua Fetii (current study) (number of collected sand flies/trapping nights). A - all traps; B - CDC miniature light traps; C - sticky traps

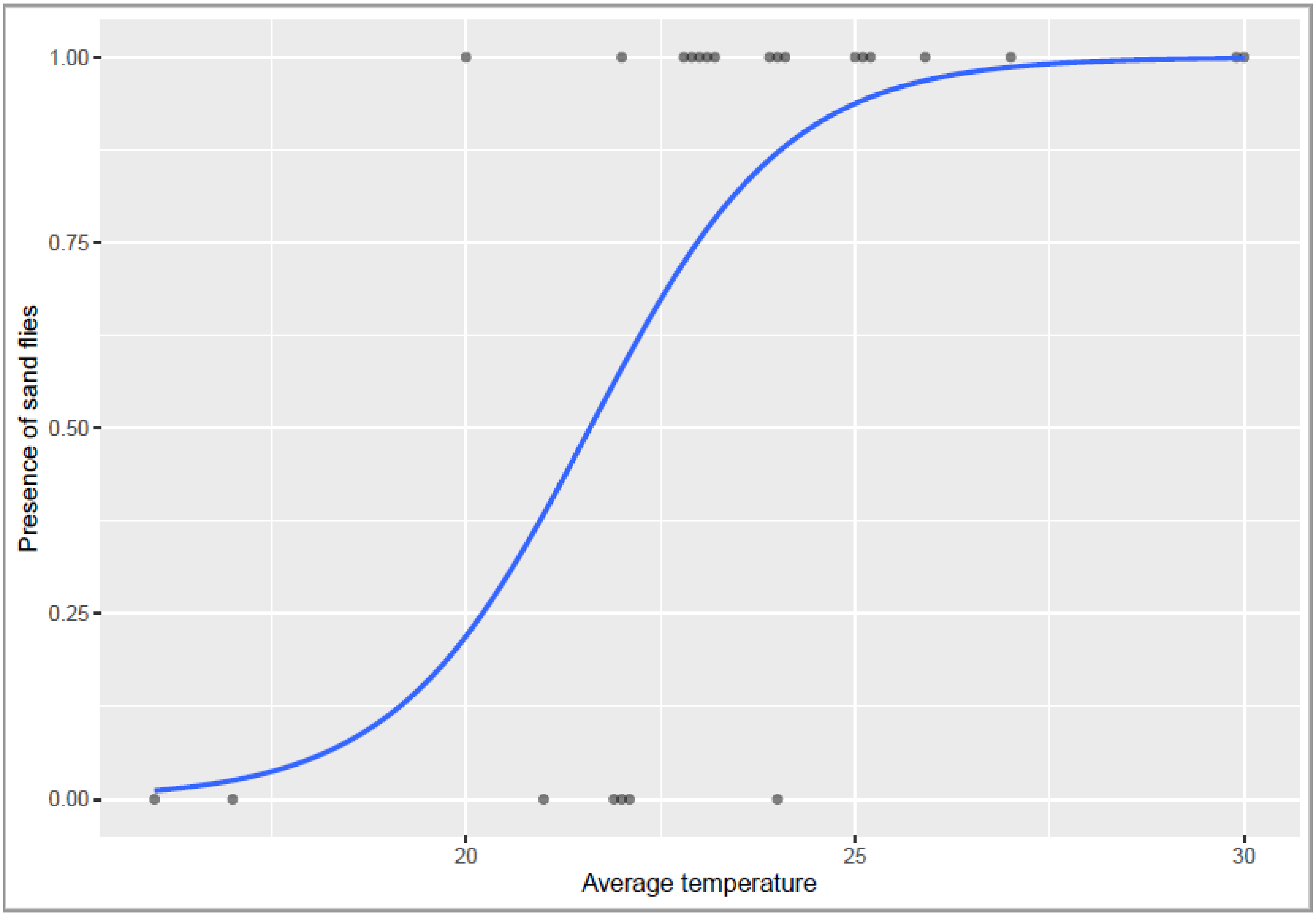

Figure 5

Logistic model of the associations between the presence/absence of sand flies and the corresponding average temperature values (p =.048)

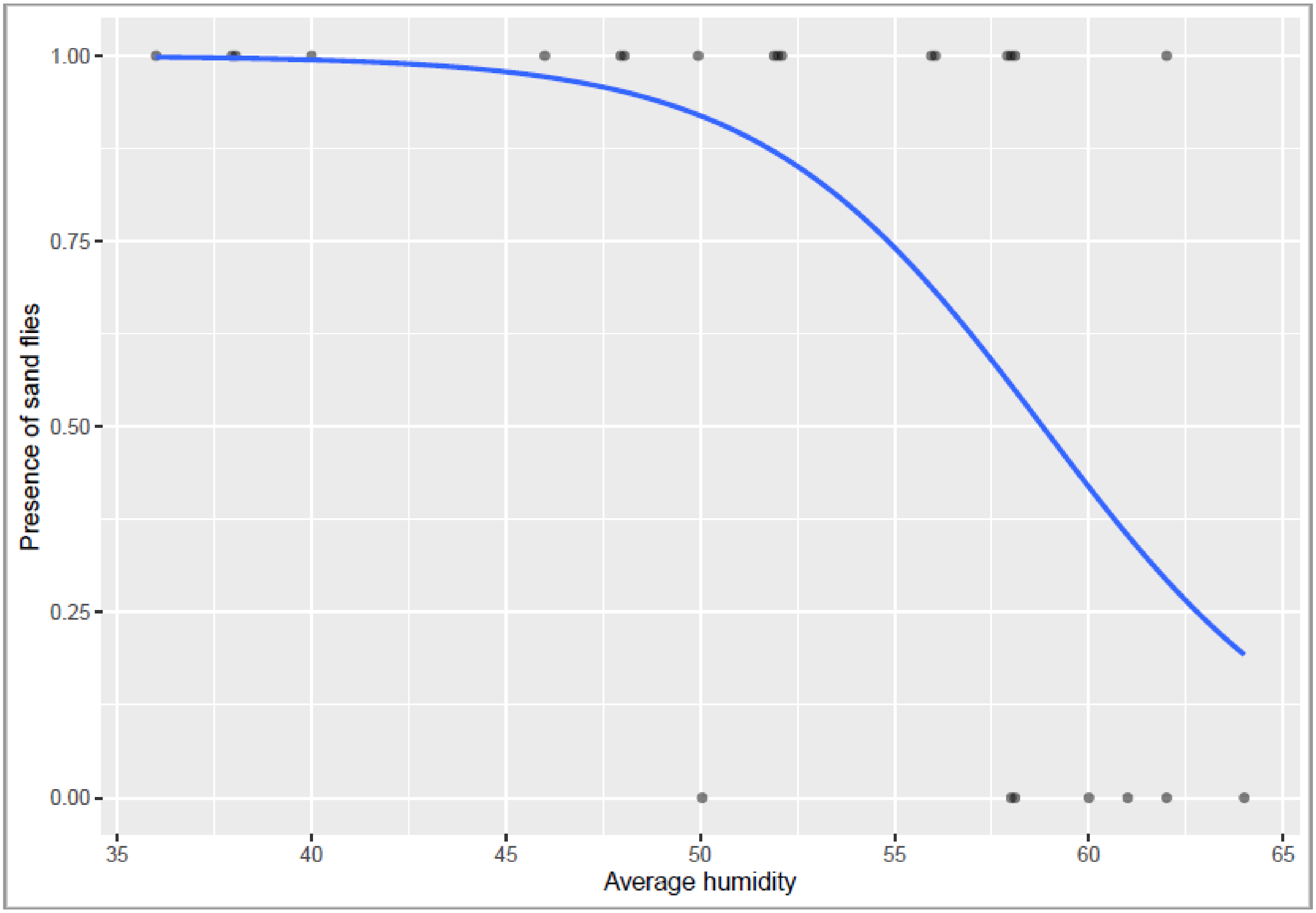

Figure 6

Logistic model of the associations between the presence/absence of sand flies and the corresponding average humidity values (p =.038)

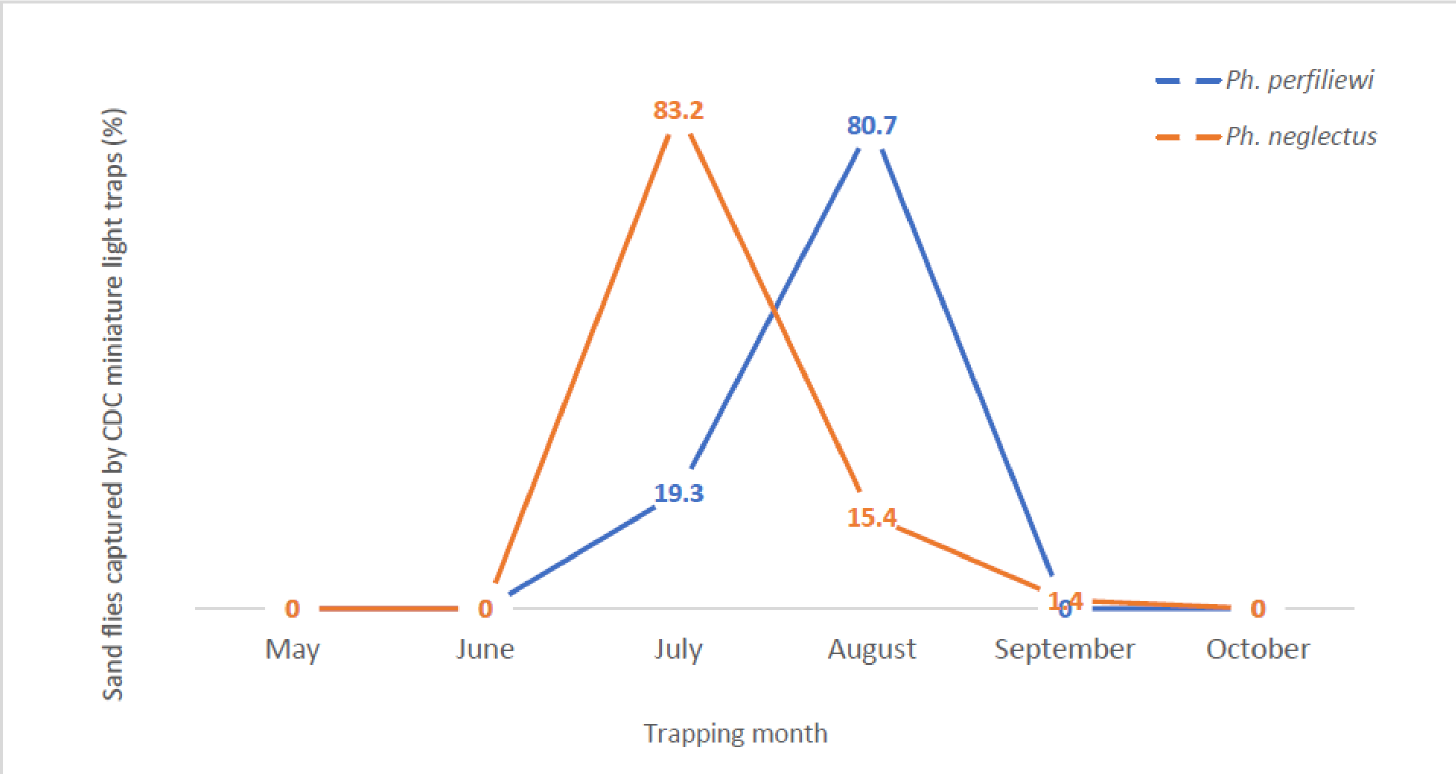

Figure 7

Relative abundance of Ph. neglectus (current study) and Ph. perfiliewi (Cazan et al., 2019b) in CDC miniature light traps

Supplementary Files

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- Table1.xlsx
- Supplementarymaterial1.xls
- graphicalabstract.jpg