Articulos

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Biología de *Enchophyllum nigrocupreum* (Hemiptera: Membracidae) asociada al "ombú", *Phytolacca dioica*, en La Plata, Buenos Aires, Argentina

Arnaldo MACIÁ
Universidad Nacional de La Plata, Argentina
arnaldo_macia@fcnym.unlp.edu.ar
Mónica L. CUNNINGHAM
Universidad Nacional de La Plata, Argentina

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Abstract: Biological aspects of the treehopper *Enchophyllum nigrocupreum* (Walker) are covered for the first time showing its association with "ombú" as a host plant. We provide information about taxonomy, distribution, association with other arthropods and seasonality in sex ratios, body weight, female gonotrophic status, and total lipid and protein content.
Keywords: Body lipids, Body proteins, Novel records, Population biology, Seasonality.
Resumen: Se dan a conocer por primera vez aspectos biológicos del membrácido *Enchophyllum nigrocupreum* (Walker) y su asociación con el "ombú" como planta hospedadora. Se provee información sobre taxonomía, distribución, asociación con otros artrópodos, variación estacional de la proporción de sexos, tamaño corporal, estado gonadotrófico de las hembras y contenido de lípidos y proteínas totales.
Palabras clave: Biología poblacional, Estacionalidad, Lípidos corporales, Proteínas corporales, Registros nuevos.

INTRODUCTION

Members of the family Membracidae (Hemiptera: Cicadomorpha), or "treehoppers", can be recognized by the extraordinary development of the pronotum, which possesses different morphological configurations, shaped as horns, spines and other prolongations in many species. They often exhibit striking colors, and many aspects of their behavior are also fascinating, such as maternal care, gregarism, communication through vibration signals and mutualism with other insects, among others (Wood, 1993; Rodríguez et al., 2004; Lin, 2006). Treehopper diversity encompasses more than 3490 species (Dmitriev et al., 2024) distributed in all biogeographic regions, although roughly half of them are endemic to the Neotropics (Remes Lenicov, 2020). The family includes representatives of high agronomic importance, mainly because they damage plant tissues by inserting their mouthparts to reach sap, and during endophytic egg-laying using the ovipositor. Other species became pests when their densities are high enough to be harmful to crops (Deitz, 1989). Some are known vectors of phytopathogens (Simons & Coe, 1958; Flasco et al., 2021). In Argentina, *Ceresa nigripectus* Remes Lenicov causes injuries in vineyards (Vicchi & Remes Lenicov, 2019) and was tagged as a potential vector of phytoplasmas causing "alfalfa witches' broom" (Meneguzzi et al., 2008, 2009).

Even though treehoppers have been employed as a model to test hypotheses in ecology, evolution, biogeography and behavior, there is a paucity of biological studies of the group in South America (but see Creão-Duarte et al., 2012; Torrico-Bazoberry et al., 2014). In Argentina, although there are publications on systematic and distribution of the family, articles on ecology are even scarcer. Life table parameters of *Guayaquila projecta* (Funkhouser) were estimated on the ornamental plant *Bougainvillea glabra* Choisy ("Santa Rita") (Linares et al., 2010) and of *Ceresa nigripectus* Remes Lenicov on its host plant *Medicago sativa* L. ("alfalfa") (Pérez Grosso et al., 2014), being both studies performed in laboratory settings. Interactions between nymphs and adults of *Enchenopa sericea* Walker and *Camponotus rufipes* (Fabricius) ants were analyzed on the bush *Caesalpinia gilliesii* (Wall. et Hook.) Dietr. ("barba de chivo") in Córdoba province (Perotto et al., 2002). Hamity et al. (2020) made a survey of fauna of Hemiptera associated to trees in an urban environment in Jujuy province and mentioned the presence of two membracid morphospecies living in *Enterolobium contortisiliquum* (Vell.) Morong ("pacará" or "timbó"). A complete list of treehopper species from Argentina and their host plants was reported by Remes Lenicov et al. (2004). The general knowledge of the family, a detailed description of external anatomy, bibliographic references about keys to the genera and species levels, and a list of the most important collections from Argentina and worldwide were summarized by Remes Lenicov (2020), who listed 140 species from 55 genera for Argentina.

Phytolacca dioica L. (Phytolaccaceae) is a deciduous and dioecious tree species commonly known as "ombú" or "bella sombra". It is native to Northeast Argentina, Uruguay, Southern Brazil, and Paraguay. In Argentina, it is widely known for its ability to provide shade in large grasslands and because it has some properties for popular medicine (Lahitte et al., 1999). It also has value as a paradigmatic tree present in many cultural manifestations of native dwellers.

In La Plata city, Buenos Aires province, Argentina, there are numerous specimens of "ombú" in the urban area, which were planted with ornamental purposes (Delucchi et al., 1993). They are mainly found in recreational wooded areas, such as squares, parks and boulevards. Nymphs and adults of treehoppers were collected by one of us (AM) on a specimen of "ombú" tree in Saavedra Park, in November 2020, and later identified by AMMRL as *Enchophyllum nigrocupreum* (Walker). As it refers to a genus distributed nearly exclusively in South America and the lack of data about the biology of the species (Strümpel & Strümpel, 2006), we aimed to obtain samples of populations and explore some traits of its ecology. In the present work we provide information about the following biological aspects of *E. nigrocupreum*: anatomical features and distribution, association with the host plant, interaction with ants, identification of natural enemies, seasonality in sex ratios, in body weight, in reproductive parameters, and in total body lipid and protein content. We raised the following specific hypothesis: 1: Are there temporal changes in sex ratios in *E. nigrocupreum*? If there is a variation in the proportion of sexes, this could be evidence of some factors acting differently on males or females. 2: Are there changes in body weight across seasons of the year or across local populations (= between individuals from different trees)? If such seasonal changes do exist, this would provide evidence of seasonality in body size in *E. nigrocupreum* populations. 3: Is there a homogeneous distribution of percentage of gravid females across the year, or there are seasonal changes in the average gonotrophic status of female *E. nigrocupreum*? If such seasonal changes do exist, this would be evidence of seasonal peaks in oviposition activity. 4: Is there a homogeneous distribution of the total body lipid and protein content throughout the year, or are there seasonal changes in total lipid and proteins in *E. nigrocupreum* populations? If such seasonal changes do exist, it would be evidence of moments of accumulation of energy reserves and tissue building to cope with unfavorable environmental conditions. These questions were analyzed with the general objective of showing some overall characteristics of autoecology of *E. nigrocupreum*.

MATERIALS AND METHODS

Insect sampling

To explore characteristics of *E. nigrocupreum* populations and their association with host plants and other arthropods, 77 “ombú” trees were identified and numbered in different places of the localities of La Plata, City Bell, and Manuel B. Gonnet, Buenos Aires province, Argentina. For each tree, sex of the plant and the presence of *E. nigrocupreum* were recorded between November 22, 2020 and May 30, 2023 (59 sampling dates). From November, 2020 to February, 2022 samples were collected irregularly and used for preliminary observations. Insects were collected by hand on leaves and branches from the ground level to a maximum height of 2.20 m. Samples were preserved in vials with a fresh “ombú” leaf from the same tree where they were collected; vials were labeled with sample provenance (place and tree number). Ants that were observed interacting with treehoppers were also occasionally caught. Samples were processed on the same date of collection, maintained *in vivo* at 8 °C for less than five days, or kept in alcohol 70 % until processing. Sampling was carried out in a non-systematic way because catches were not repeated periodically for each tree. Instead, sampling was conducted on trees with higher observed number of treehoppers to ensure representative numbers of insect populations.

Identification was based on the original description, and external anatomy and genital structures (Dietrich & McKamey, 1995; Strümpel & Strümpel, 2006: 341, 359; Flórez et al., 2015). Voucher specimens of adults and nymphs were deposited in the Museo de La Plata entomological collection (MLP).

Insect dissection

To analyze whether seasonality in sex ratios (hypothesis 1), in body weight (hypothesis 2) and in the reproduction dynamics (hypothesis 3) is present in subpopulations of *E. nigrocupreum* (defined herein as individuals coexisting on the same tree in a given moment), we examined 537 individuals from 49 “ombú” trees between February, 2022 and March, 2023.They were dissected under a binocular stereoscope Zeiss Stemi SV6 at 8X provided with a micrometric scale. Female abdomens were teased apart along the ventral midline and, according to the ovariolar stage of development, were classified either as gonoactives (follicles with evident yolk) or gonoinactives (follicles at rest, without noticeable yolk). In gonoactive females, the number of oocytes with yolk was recorded.

Lipid and protein content quantification

To determine the seasonal fluctuation in the total corporal lipid and protein content in subpopulations of *E. nigrocupreum* (hypothesis 4), 553 individuals were caught on seven “ombú” trees (two or three replicates on each tree) on the following sampling dates: 15 and 21/6/2022, 28/10/2022, 21 and 23/2/2023 and 13 and 14/5/2023. Then their gender was determined, and after a period of less than 24 h. at 8 ºC, weighed individually *in vivo* to the nearest 0.0001 g in a Shimadzu AUY 220 scale. Treehoppers were immediately frozen (-18 ºC) in plastic vials without preservative liquids. These insects were used to quantify the amount of lipid and protein of males and females in different seasons of the year, measured as mg per g of body weight.

Each group of insects was homogenized with a Potter-Elvehjem homogenizer in phosphate potassium buffer (50 mM, pH 7.4) in a ratio of 30 µl of buffer per 0.004 g of sample, adding a 1:1000 v/v protease inhibitor cocktail (Sigma Chemicals, Saint Louis, Missouri, USA). The homogenate was centrifuged at 10.500 g for 20 min at 4 ºC. After discarding the particle pellet from the centrifugation, the supernatant volume was measured. From the latter, 20 µl were extracted to quantify proteins and the rest was allocated to analyze lipids. These samples were stored at -70 ºC until processed. To obtain total lipids from all samples the extraction method by Folch et al. (1957) was used. Quantification of lipids was made by gravimetric analysis and estimation through measuring their weight. To achieve this, total lipid samples were subjected to a stream of N_2 to evaporate solvent until dryness. Once evaporated, samples were suspended again in 1000 µl of mixed chloroform: methanol 2:1 (v/v). From each suspended sample 250 µl were transferred to small glass dishes, the solvent was dried with a stream of N_2 and finally weighed in a Mettler-Toledo scale. The weight obtained was subsequently referred to the volume of the total sample.

The total protein concentration of each group was measured colorimetrically by the method of Lowry et al. (1951).

Statistical analysis

To establish sex ratios (SR) within populations of *E. nigrocupreum* as well as their fluctuation, we estimated the response variable SR= $N_{males} / N_{males} + N_{females}$ (Ancona et al., 2017). The likelihood ratio chi-squared statistic, G^2 was estimated under the null hypotheses of a sex proportion= 0.5:0.5. SR was tested for departure from equality for each month of sampling. To evaluate if average body weight differs amongst individual trees and to compare body weight fluctuation across seasons of the year, we first checked for normality and homoscedasticity of raw data with the Kolmogorov and Shapiro-Wilks test, respectively ($p > 0.05$). After finding a significant departure from a normal distribution and verifying unequal variances of body weights, the non-parametric Kruskal-Wallis test was used, followed by medians discrimination as described by Conover (1999). This test was also applied to the variable potential fecundity across trees. Total lipid and protein body contents across seasons of the year were analyzed as response variables with parametric one-way ANOVA with seasons of the year as the source of variation, followed by Bonferroni tests to separate treatments means with $\alpha = 0.05$ and to control for the number of multiple comparisons. All tests were separately performed for males and females. Statistical tests were processed with Infostat 8.0 (Di Rienzo et al., 2017).

RESULTS

Taxonomy and distribution

The neotropical genus *Enchophyllum* Amyot & Serville belongs to the tribe Membracini, the most diverse of the subfamily Membracinae, with 15 genera and 203 described species (Dmitriev et al., 2024). Based on shared anatomical characters -mainly the pronotum morphology and pattern of coloration- *Enchophyllum* was classified as closely-related to *Enchenopa* Amyot & Serville due to similarities in the shape of the pronotum and extension of its carinae, but differing from the latter in having a distinct pronotal horn, and the arrangement of pronotal carinae that terminates before or above the humeral angle (McKamey, 2022). Strümpel & Strümpel (2006) revised and redefined *Enchophyllum,* providing a key for the identification of adults of 11 recognized species, and restricted the genus to those species with only one lateral carina and without any carinae on the metopidium. Although the shape of the pronotum and color pattern are the most distinctive features, the male and female genital structures as well as the proportions of the head and pronotum were additional characters used to distinguish between individual species with very similar external morphology. Regarding the morphology of immatures, Strümpel and Strümpel (2006) recognized them as belonging to this genus by having the abdominal scoli all subequal in length and with a uniform or mostly uniform gray or white wax-like covering although some species of *Enchenopa* also share this feature (McKamey, 2022).

Following the characteristics stated for adults and nymphs by Strümpel & Strümpel (2006) and complemented by McKamey (2022), adult *E. nigrocupreum* are identified by its conical, broad and compressed pronotal horn pointing anteriad, the integument black to cupreus brown, and the pronotum with three pale spots: one broad frontal spot extending onto the metopidium as far as the base of the horn, a second large spot in the middle of the dorsum, and a third one posterior to the second, which forms a subapical band -in all individuals such spots were white and none showing a yellowish coloration as consigned by Strümpel & Strümpel (2006). The forewings are brown and blackish towards the base; the tarsi are brown. It is also distinctive its dorsum profile from the tip of the horn to the posterior apex slightly undulating. The male genitalia with the posterior arm of the U-shaped aedeagus much larger than the anterior one, have minute denticles covering 1/3 of the dorsal and lateral surfaces. The shank of the style is slender and recurved and ends truncate apically (Fig. 1a). Immatures (Fig. 1b) have the integument coated by a whitish secretion through which fairly fine and erect setae protrude, and with the dorsal margin of the pronotum, the posterior margins of the wing pads, and parts of the abdomen and legs, blackish. The abdominal terga III through VIII each have a pair of long, suberect, submedial, blackish brown scoli. The subequal size of the abdominal scoli has been understood as a generic feature that usually differs from the immature of *Enchenopa*, which may be the same or have the scolar size increasing posteriorly (Strümpel & Strümpel, 2006; McKamey, 2022).

To date, two species have been recorded from Argentina: *Enchophyllum cruentatum* Germar and *E. nigrocupreum* (McKamey, 1998; Strümpel & Strümpel, 2006); a third species, *Enchophyllum imbelle* Stal (catalogued in McKamey, 1998 and Remes Lenicov, 2020) was transferred to Membracini *incertae sedis* by McKamey (2022).

Regarding the geographical distribution, the precedent scientific literature mentions the presence of *E. nigrocupreum* in Argentina in a single locality in Misiones province, in addition to Brazil, Paraguay (Strümpel & Strümpel, 2006; Vieira da Silva, 2017), and Colombia (Flórez et al., 2015). Additionally, there are some anonymous contributions to different websites showing pictures of the species. There is a striking correspondence on a large scale of distribution areas of *E. nigrocupreum* and *P. dioica* (Instituto de Botánica Darwinion, 2024; GBIF, 2024).

Until now, no records in literature have mentioned the plant species on which *E. nigrocupreum* was collected, making this contribution the first one to document its host plant.

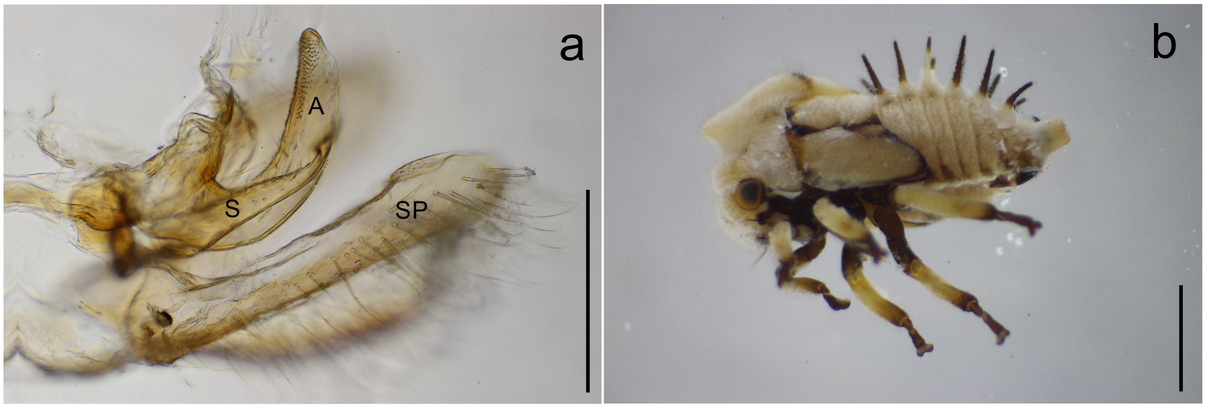

Figure 1.
Genitalia and immature of *Enchophyllum nigrocupreum*.
a. Male genitalia. b. Fifth nymphal stage. References= A: aedeagus, S: style, SP: subgenital plate. Scale bars= a: 0.5 mm, b: 1 mm.

Observations on the biology

All specimens were found on female and male individuals of *P. dioica* and none on other plant species from the surroundings. A total of 1378 adults of *E. nigrocupreum* (438 males and 940 females) were collected between 2020 and 2023. They occupied the upper and lower surface of leaves, petioles and green thin stems with or without lignified bark (Fig. 2a) but were not observed on the trunk or woody branches. Nymphs aggregated in groups of mixed instars on stems and bases of leaves. While sampled, nymphs exhibited little to no movements. The adults, which also had remarkably sedentary habits, sometimes exhibited displacement by walking or launching in flight avoiding capture. Some specimens were observed copulating in November 2021 (Fig. 2b). Fifteen to 20 eggs were laid by females into the plant's tissue and the egg clutch was covered with white foamy secretions (Fig. 2c), which gets a rubbery texture a few days after oviposition. Egg masses were laid on the lower surface of leaves, on the mid-rib, generally at 1/3 of the distance between the basis of the leaf and its apex, or at 2/3 of that distance (Fig. 2d). Each egg is oval-shaped with one extreme sharper than the opposite. Egg clutches were not protected by adults.

In August, 2021 and November, 2022, 16 teneral adults were noticed due to their whitish tegument.

Associated arthropods

In seven females and one male, mites identified as *Charletonia* sp. (Trombiculidae: Erythraeidae) were found attached to the insect body or to a wing vein (seven individuals with one mite and one with two) and covered by the fore or hind wing or under the pronotum

(Fig. 2d). They were found from March to May, 2022 and January, 2023.

Enchophyllum nigrocupreum nymphs were often interacting with ants identified as *Camponotus mus* Roger, *Brachimirmex* sp. and *Pseudomyrmex* sp., which obtained honeydew from them (Fig. 2e). Treehoppers were attended by a single ant species in particular on each occasion where co-occurrence was observed.

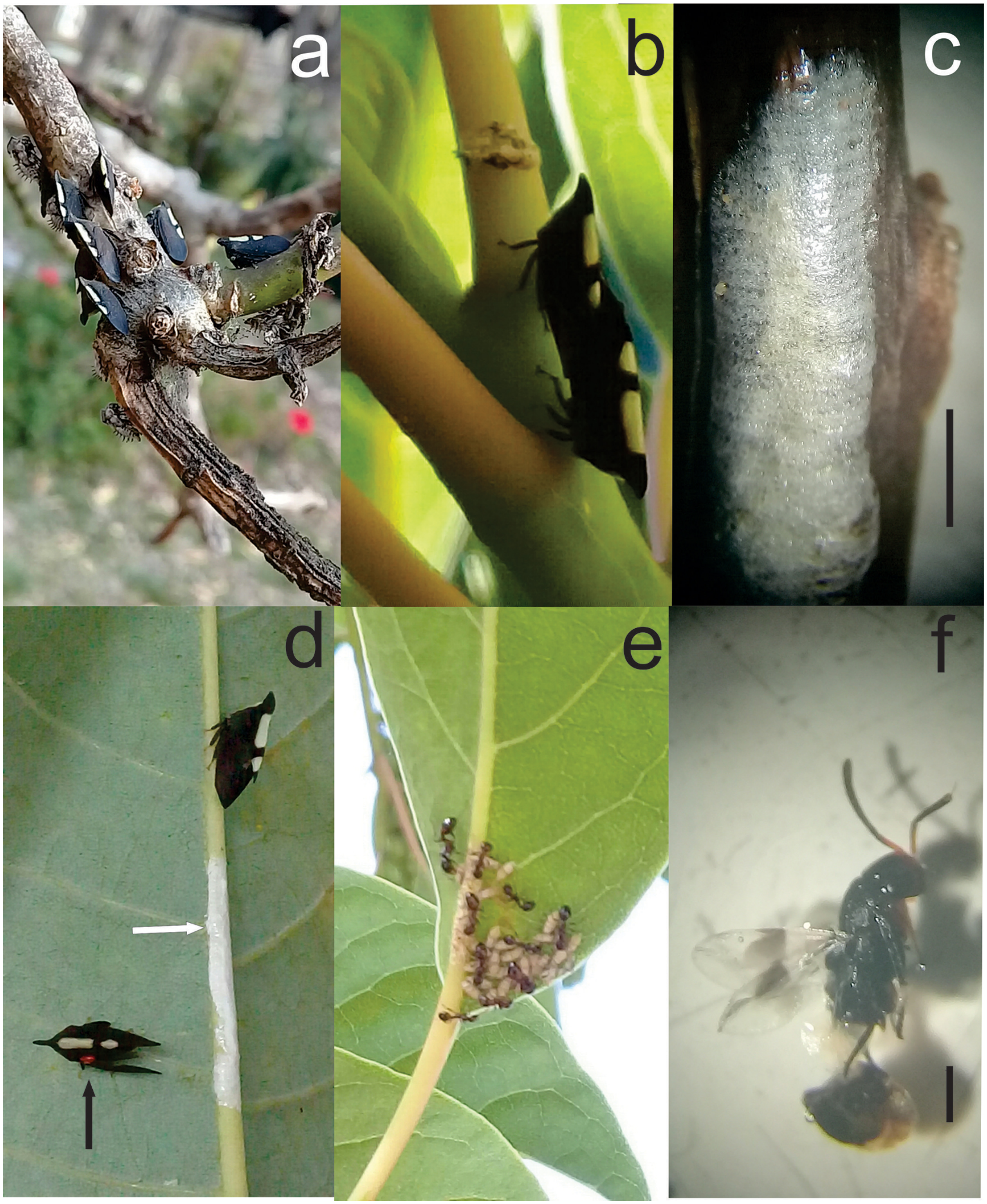

Figure 2.

Adults, nymphs and eggs of *Enchophyllum nigrocupreum* and their associated arthropods.

a. Adults and nymphs. b. Mating adults. c. Egg froth. d. Egg mass (white arrow) on lower surface of a leaf near its mother and another individual with an attached *Charletonia* sp. mite (black arrow). e. *Brachimyrmex* sp. ants attending nymphs. f. *Prionomastix* cf. *caralis* adult from a dissected female of *E. nigrocupreum* (bar: 1 mm). Scale bars= c, f: 1 mm.

In December, 2022 and January, 2023, five females were parasitized by one individual of *Prionomastix* cf. *caralis*

(Hymenoptera, Chalcidoidea, Encyrtidae) (Fig. 2f). Four of them have a pupa inside the abdomen, and in another one a fully developed adult was recovered following the dissection. No external evidence of parasitism was seen in the treehopper body.

Sex ratios

Sex ratios in *E. nigrocupreum* were significantly biased to a female preponderance considering the total of individuals (pooling for sampling sites and dates) in which proportions between sexes were inspected (804 females and 345 males, G^2= 188.58, 1 d. f., p< 0.001). During all months of sampling females outnumbered males, although in July 2022 (18 females and 9 males, G^2= 3.06, 1 d. f., p= 0.080), March (16 females and 9 males, G^2= 1.99, 1 d. f., p= 0.159) and May 2023 (83 females and 66 males, G^2= 1.94, 1 d. f., p= 0.163) there was a non-significant deviation from the null hypotheses of a 0.5:0.5 sex ratio (Fig. 3).

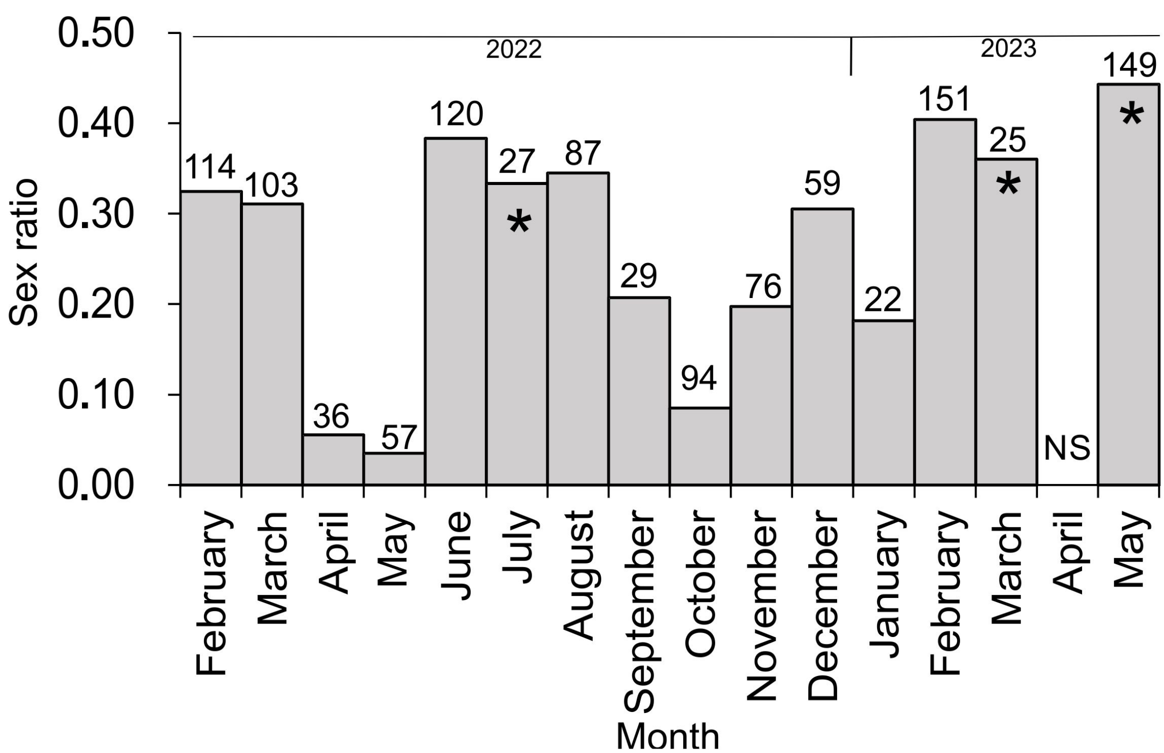

Figure 3.

Monthly fluctuation in sex ratios (SR= Nmales / Nmales + Nfemales) of *Enchophyllum nigrocupreum* from February 2022 to May 2023.

Asterisks show months in which sex ratio was not significantly different from 1:1. Numbers on columns show sample size. NS: not sampled.

Body weight

Weight of females ranged from 0.00120 to 0.01920 g (mean ± SD: 0.0131 ± 0.002 g, n= 346) and of males from 0.00520 to 0.00990 g (mean ± SD: 0.0079 ± 0.001, n= 174). In both sexes, the variable body weight rendered non-normal and variances were heterogeneous (males: Komogorov d= 0.417, $p < 0.05$; Shapiro-Wilks W= 0.672 p< .05; females: d= 0.431, $p < 0.05$; W= 0.637, $p < 0.05$). Thus, non-parametric comparisons by ranks of body weights amongst individual "ombú" trees were performed. With the aim of obtaining sufficient sample sizes, insects for this analysis came from five different "ombú" trees in the case of males and from six "ombú" trees in the case of females. There were non-significant differences between medians of body weights in males across individual trees (Kruskal-Wallis H= 2.67, 4 d. f., p= 0.615) and in females (H= 6.29, 5 d. f., p= 0.28).

We tested for the effect of season of the year (spring, summer, autumn and winter) on median body weight of *E. nigrocupreum* of both sexes and found a significant effect of the main factor in males (n= 174, H= 31.55, 3 d. f., $p < 0.05$) (Fig. 4). For this sex, median body weight did not differ between winter, summer and spring; median body weight in autumn was significantly higher than in winter and summer but not from spring. There was also a significant seasonal effect on median body weight of females (n= 335, H= 38.19, 3 d. f., $p < 0.05$) (Fig. 4). Multiple comparisons of median weight showed that body weight in spring was significantly higher than in summer and winter; median body weight in autumn was higher than in summer; weight in spring did not differ from autumn which in turn did not differ from winter.

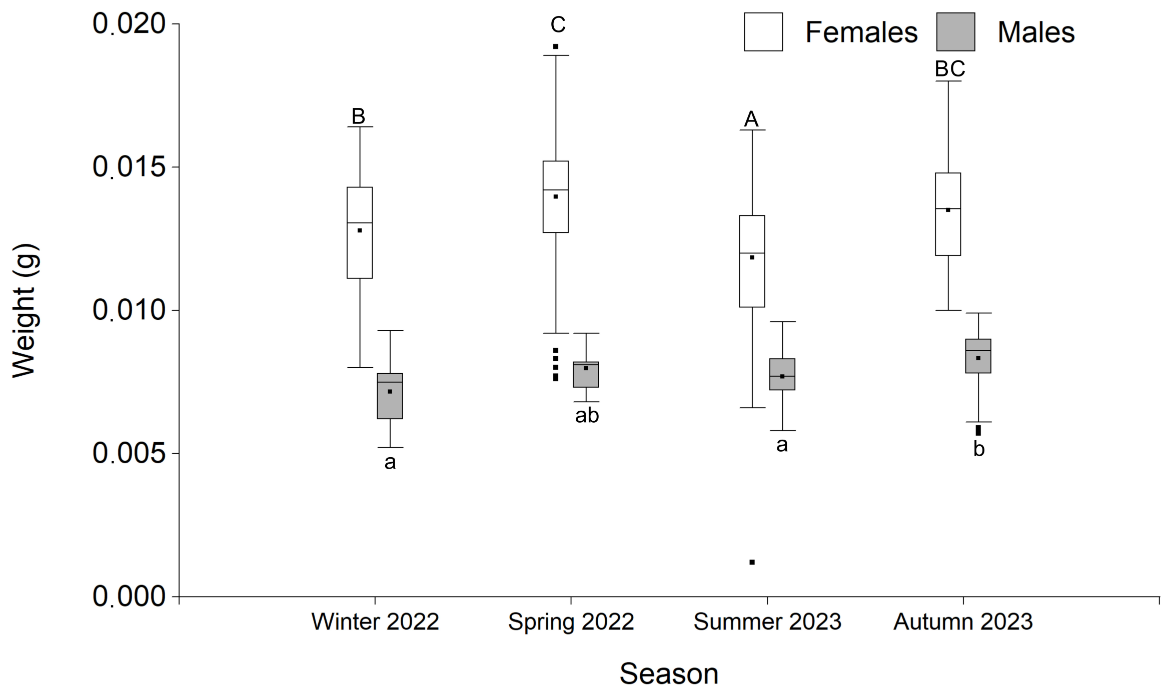

Figure 4.

Mean, median, 0.05, 0.25, 0.75, 0.95 quantiles and extreme values of body weight of males (gray boxes) and females (white boxes) of *Enchophyllum nigrocupreum*.

Different letters indicate significant differences between groups (males: lowercase letters, females: capital letters).

Body weight showed subtle temporal fluctuations. Females were heavier in spring and autumn, and males in autumn. The reasons for this size variation are unknown, but as there were non-significant differences in insect weights between trees, the variability may be attributed to factors other than specific differences between individual host plants. The number of developing eggs in female´s ovaries is a measure of potential fecundity, and it did not differ between individual trees either. Potential fecundity was within the range of other membracid species in field conditions. For example, Mitchell & Newsom (1984) reported for *Spissistilus festinus* (Say), a treehopper pest from southern North America, 22 ± 9 eggs/female and a maximum of 61 eggs in a single female, which constitutes very similar numbers to our records for *E. nigrocupreum*. However, measures of fecundity in *S. festinus* vary according to seasons and host plants, because the species is polyphagous. The percentage of gravid females of *E. nigrocupreum* reached a peak during spring, a fact consistent with the seasonal variation for *S. festinus* described by Sisterson et al. (2023). It is suggested that *E. nigrocupreum* is iteroparous [several or many reproductive events during lifetime (Begon et al., 1990)] and that a peak in ovipositions occurs in spring, resulting in overwintering of the populations in the adult and nymphal stage. A decrease in lipid was evident in females collected in winter, perhaps reflecting the mobilization of energy reserves during the harsh season. The findings of teneral adults in winter and spring suggest the existence of at least two generations in field conditions. Moreover, this is corroborated by the observation of all stages of development coexisting during all seasons. Finally, a notable increase in proteins in both sexes during winter compared to other seasons, indicating augmented tissue development. Additional research is crucial to interpret why protein levels are higher in winter, but *a priori* some reasons could be related to survival, through storage of protein resources, or adaptation to low temperature. Elevated levels in body proteins translate into preservation and stabilization of different macromolecules, as was shown for the larval stage of the gall fly *Eurosta solidaginis* (Fitch), an insect extensively used as a model for the study of cold tolerance. In this species, as in *E. nigrocupreum* females, a 1.5-2.0-fold increase in winter was observed in proteins compared to values for September (Hahn & Denlinger, 2007; Zhang et al., 2011). Furthermore, accumulation of protein is important during reproduction, a process studied in adults of the lady beetle *Hippodamia convergens* (Guerin) where proteins seem to play a relevant role during overwintering and reproduction in spring (Mercer et al., 2020).

In summary, although seasonal fluctuations in body size, female gonotrophic status, and lipid and protein levels are expected, populations of *E. nigrocupreum* remain active year-round, perhaps favored by a high stability in the habitat occupied by local treehopper populations. "Ombú" trees provided a constant supply of resources (food, oviposition substrate, refuge) to treehoppers and in this sense, a major determinant of their life history.

Lin (2006) classified treehopper species according to their social behavior in three categories: solitary, aggregated and subsocial species. Aggregation of nymphs and adults of *E. nigrocupreum* fits the second category, because many groups of nymphs, adults, or both were frequently observed, but neither males nor females were observed protecting egg clutches or nymphs. As was pointed out for several species of the family, gregarism enhances ant mutualism (Wood, 1993; Perotto et al., 2002; Lin, 2006).

The collecting method employed in the present work is efficient if compared with other common means of obtaining samples, such as yellow sticky cards and light traps (Cabral et al., 2020). Manual collection is valid for a semi-sedentary insect and for a system where no other membracid species were present, because other methods are more adequate for estimating biodiversity. Although samples were extracted at different time intervals for each "ombú" tree (except for lipid and protein extraction), they were not necessarily representative of population density or abundance. Therefore, our study cannot be used to accurately estimating total population levels. Beyond population abundance, other interesting ecological issues could be explored using *E. nigrocupreum* as a research model such as colonization and extinction in trees as islands as in Wood & Dowell (2022), vibrational communication between individuals, and population regulation and cycles. *Enchophyllum nigrocupreum* was not indicated as a harmful organism because it does not visibly affect its host plant nor transmit any pathogen as far as we know, and consequently is not of phytosanitary or economic importance.

The present research provides some basic biological data about an insect species that may serve as a benchmark for regional biodiversity studies. Because this treehopper is closely allied with a tree typical from temperate plains from the Southern Cone, it might serve as an important biological indicator of ecosystem health as well.

Female gonotrophic status

There were gravid females all along the sampling period, although the proportion of gravid females amongst all females fluctuated across time. There were a total of 185 gravid females from among 550 dissected females, or 33.6 %. Gravid females had between 1 to 62 eggs retained in the reproductive tract, and there was an average (± SD) of 20.74 (± 11.87) eggs per female. There were non-significant differences in the number of developing oocytes of gravid females between individual "ombú" trees (Kruskal-Wallis H= 13.63, 20 d. f., p= 0.85). The percentage of gravid females in the population (data pooled across trees) attained a maximum during spring (September to December); and there was a decrease in the percentage of gravid females from spring to winter (June to September) (Fig. 5). In winter, although the percentage of gravid females was the lowest in the populations, there were still 16 of 74 females with 3 to 51 oocytes with yolk in the ovaries.

Lipid and protein content

In males, means of total lipid content did not differ between seasons of the year ($F_{3,5}$= 1.54, p= 0.31) (Fig. 6). Means of total protein content were significantly heterogeneous across seasons ($F_{3,5}$= 15.47, p= 0.006) (Fig. 7); males protein content was higher in winter than in the other seasons, which in turn did not differ significantly.

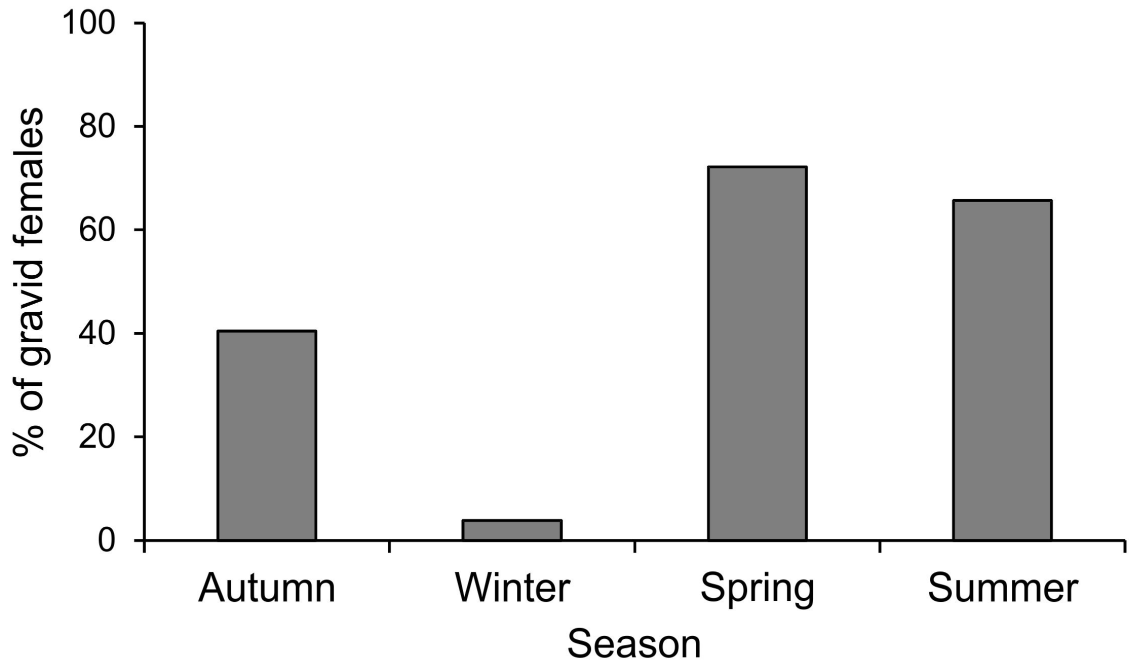

Figure 5.
Seasonal fluctuation of percentage of gravid females of *Enchophyllum nigrocupreum* from autumn 2022 to winter 2023.

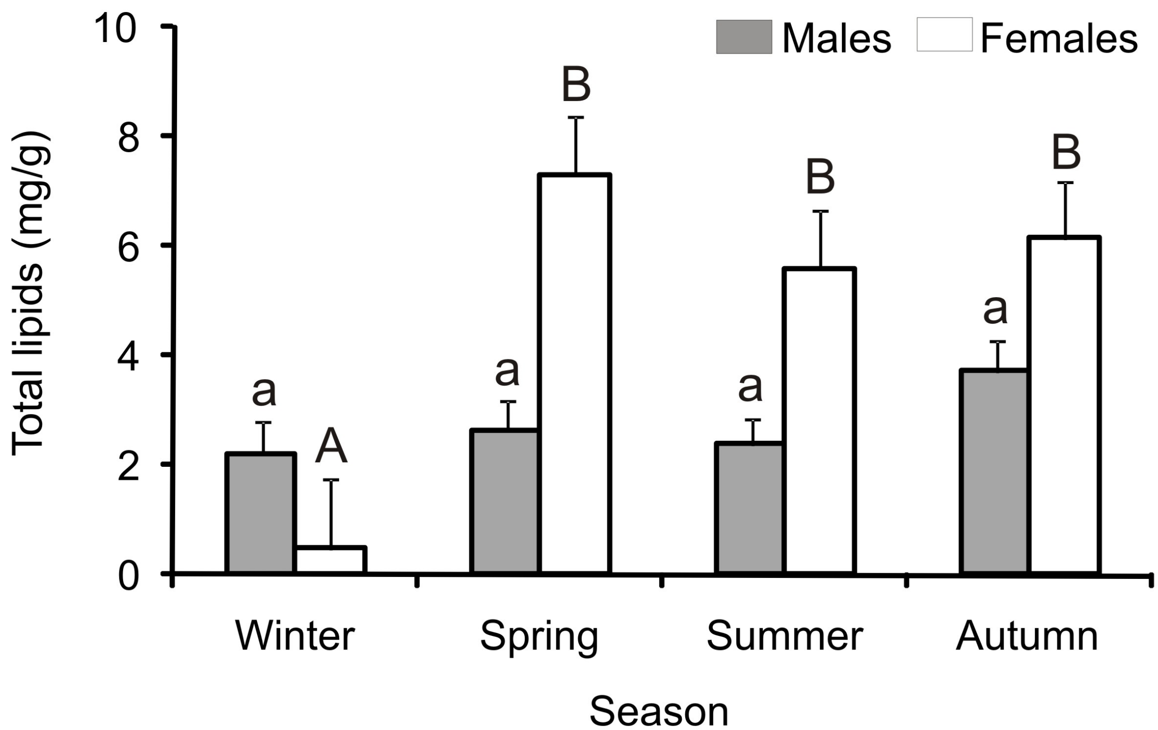

Figure 6.

Seasonal fluctuation of mean (±SEM) lipid content of males and females of *Enchophyllum nigrocupreum*.

Different letters indicate significant differences between groups (males: lowercase letters, females: uppercase letters).

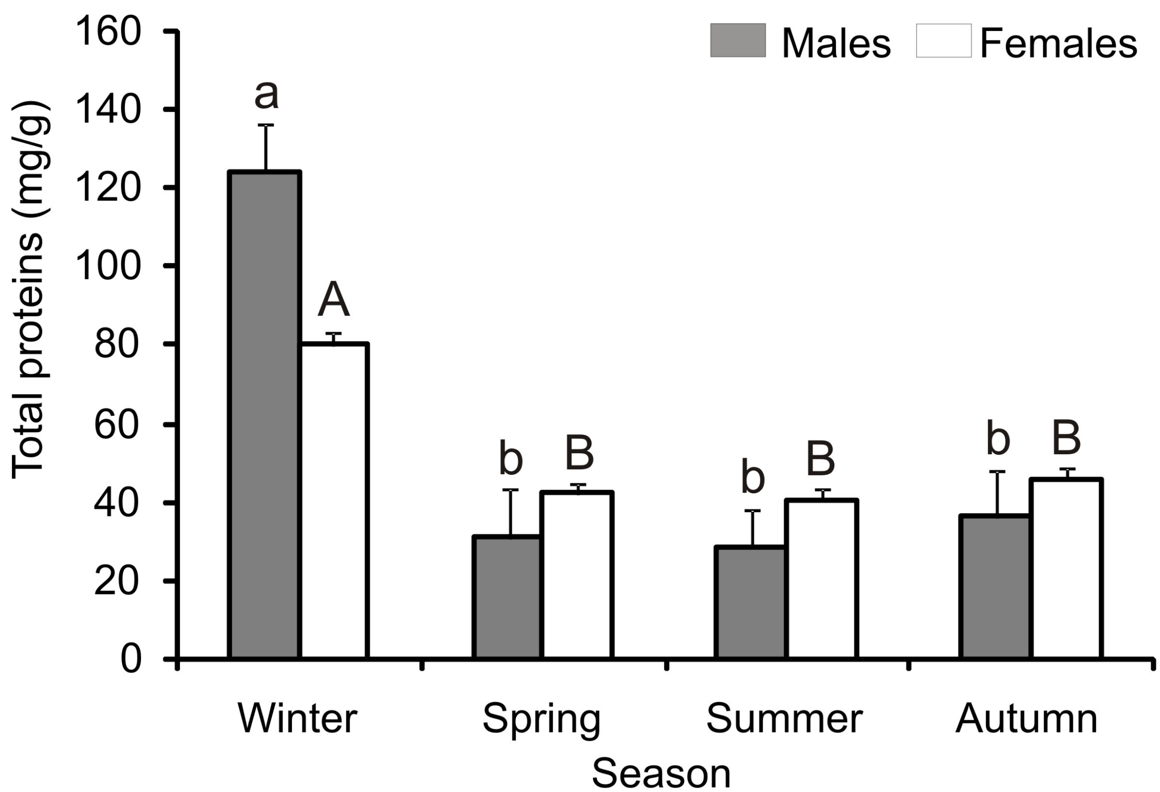

Figure 7.

Seasonal fluctuation of mean (±SEM) protein content of males and females of *Enchophyllum nigrocupreum*.

Different letters indicate significant differences between groups (males: lowercase letters, females: uppercase letters).

In females, the effect of season of the year was significant both on total lipid ($F_{3,7}$= 6.08, p= 0.023) (Fig. 6) and protein ($F_{3,7}$= 32.74; p= 0.0002) (Fig. 7) contents. For this sex, lipid content was lower in winter than in spring, summer and autumn, which means were homogeneous; on the other hand, total proteins showed the opposite trend, as mean content was higher in winter than in the other seasons, which were not significantly different.

Variability around means was accounted in a higher degree for the response of protein content than for lipid content in both sexes by the ANOVA model and was higher for females than for males as well (males: R^2_{lipid}= 0.48, $R^2_{protein}$= 0.90; females: R^2_{lipid}= 0.72, $R^2_{protein}$= 0.93).

DISCUSSION

Phytolacca dioica is the natural host-plant for *E. nigrocupreum*. As "ombú" trees are a common component of urban and rural settings in temperate Argentina, it draws attention that there are not previous records of this insect-plant association. To affirm that *E.*

nigrocupreum is a monophagous species requires more field surveys and laboratory assays. Feeding specialization is present in some cases within the subfamily Membracinae (Deitz, 1975), but a great part of South American species is polyphagous (Wood, 1993). Similarly, the correspondence of the distributions of *P. dioica* and *E. nigrocupreum* deserves a search in more localities from different latitudes. This would clear up if our outcomes were valid for all populations. Additionally, in areas where "ombú" trees are native and also in places where they were introduced, such as North America, Europe, India and Australia (GBIF, 2024), it is unknown whether *E. nigrocupreum* is present, and probabilities for this would be extremely low if introductions started with "ombú" seeds.

Interaction of membracids with ants is a well-known interspecific relationship (reviewed by Buckley, 1987) in which both partners of the association obtain benefits from mutualism. Nymphs of *E. nigrocupreum* probably establish an incidental relation with at least three species of ants which were observed while obtaining fluid excreted by nymphs, thus receiving a reward for attendance, but we cannot affirm that ants confer a benefit to nymphs, such as defense against, or interference with predators or parasites, among other possible advantages (Buckley, 1987). Rather, interaction seems to be conditional or inconstant, judging by the low frequency of its presence during our field observations. Mutualism or commensalism and its impact on both interacting insects have yet to be experimentally proven. Cushman & Witham (1989) described a treehopper-ant system largely subjected to changes in the physical and biological circumstances in which they occur. Some species of *Enchophyllum* [*Enchophyllum dubium* Fowler, *Enchophyllum melaleucum* (Walker)]are tended by ants while other congeneric species are not (Lin, 2006).

According to Trjapitzin (2017), the species of *Prionomastix* are mostly parasitoids of treehoppers, and for *P. caralis* hosts are unknown. In the present study, the identity of the encyrtid *P.* cf. *caralis* was identified based on a single adult specimen; if this species is further confirmed as a parasitoid of *E. nigrocupreum*, this would be the first record of Membracidae as a host for *P. caralis*, and the first record of its presence in South America. Other individuals were recovered from adult treehoppers but the wasp was in the pupal stage. Members of *Prionomastix* attack the hosts and develop until the emergence through the abdomen´s tegument (Godoy et al., 2006). This may be true for *E. nigrocupreum*; otherwise, the adult stage could represent a dead-end host for the parasitic wasp if it is not able to rupture the abdomen, or if *E. nigrocupreum* is an occasional host.

Charletonia spp. mites are ectoparasites of insects of various orders (Southcott, 1991). There are no previous records of members of Membracidae as hosts for these acari, or of its presence in Argentina. Bassini-Silva et al. (2022) recorded the exchange of individuals between gregarious psocopterans, so in the case of *E. nigrocupreum*, the habit of living in groupsmay facilitate parasitism by *Charletonia* mites.

Regarding patterns of temporal variations in population features, we corroborated a seasonal effect in some variables important for the life history of *E. nigrocupreum*, namely sex ratios, mean body weight, proportion of gravid females and total protein and lipid content in both sexes.

Females were more abundant than males during all of the sampling period. A sex ratio of 0.5:0.5 was detected in July, 2022, and March and May, 2023. Neither males nor females were seen protecting nymphs or eggs masses, and mobility seems to be the same in both sexes, thus the probability of capture was similar for males and females. Therefore, female preponderance would not be biased, and can arise from intrinsic reasons (differences between numbers of each sex at birth or differential mortality at the nymph or adult stage). Female preponderance might be a stable strategy for the species, as there was not a correspondence between the same months of different years with an equal sex ratio. Perhaps this constitutes an advantage for males because if they are less numerous than females, they have more opportunities to mate with potential partners (Ancona et al., 2017).

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Author notes

arnaldo_macia@fcnym.unlp.edu.ar

Arnaldo MACIÁ, Mónica L. CUNNINGHAM

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