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FIRST BIOLOGICAL DATA OF *CERESA NIGRIPECTUS* (HEMIPTERA: MEMBRACIDAE), A COMMON TREEHOPPER ON ALFALFA CROPS IN ARGENTINA

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ABSTRACT

Alfalfa (*Medicago sativa* L.; Fabaceae) is essential for argentinean agricultural development, but diseases like "witches´ broom», caused by the ArAWB phytoplasma, are limiting factors for the production of this crop. Insects that visit alfalfa have been poorly studied. The treehopper, *Ceresa nigripectus* Remes Lenicov (Hemiptera: Membracidae) is very frequent in alfalfa crops; ArAWB was detected in several individuals of this treehopper, but the ability of this species to transmit the pathogen has not yet been demonstrated. The Membracidae family is a poorly studied group in Argentina. No data on the biology of *C. nigripectus* are available, and therefore the aim of this contribution is to provide information on its behavioral and biological parameters as a first step toward its control. The studies were conducted in the laboratory under controlled conditions. *Ceresa nigripectus* populations were maintained on healthy alfalfa plants; the treehoppers were checked daily, the occurrence of molts was noted, and diverse aspects of nymphal and adult behavior were also recorded. A life table was built combining the observational data from 7 cohorts. Oviposition sites were characterized by dissecting plants and through staining methods. *Ceresa nigripectus* could complete its life cycle entirely by using only alfalfa as a host. Females do not tend their broods. Eggs are laid individually and rarely 2 or 3 were found in the same slit. Eggs were located in the stems, in the nodes (in the axils of the leaves) or in the internodal space, between the epidermis and cortical tissues. The pre-ovipositional period was about 6 days, and the average duration of the egg stage was 20 days. The duration of nymphal period was variable, between 32 to 63 days, with a mean of 45 days. Two periods of high mortality were recognized, i.e., in the first instar, and during the second week of the adult stage. Longevity of adults averaged 61 days, but some individuals survived up to 202 days; females lived longer than males. In laboratory colonies, the sex ratio was about 1.3:1 females:males. All the developmental stages of the treehopper were tended by the ant *Camponotus punctulatus* Mayr. Data regarding behavioral aspects and new distributional information are also provided.

Key Words: life cycle; behavior; alfalfa diseases; phytoplasma

RESUMEN

La alfalfa (*Medicago sativa* L.; Fabaceae) es esencial para el desarrollo de la agricultura argentina y enfermedades como la "escoba de bruja", causada por el fitoplasma ArAWB, son un factor limitante para la producción del cultivo. La fauna que visita este forraje ha sido escasamente estudiada en Argentina. *Ceresa nigripectus* Remes Lenicov es muy frecuente en alfalfa; estudios de laboratorio verificaron la presencia del ArAWB, pero su capacidad para transmitir la enfermedad todavía no ha sido demostrada. Membracidae es un grupo poco estudiado en el país, no hay datos sobre la biología de *C. nigripectus* y por ello el objetivo de esta contribución es proporcionar información sobre sus parámetros biológicos y comportamentales. Los estudios se realizaron en laboratorio bajo condiciones controladas. *C. nigripectus* se mantuvo sobre plantas sanas de alfalfa y para estudiar el ciclo de vida, los individuos fueron controlados diariamente observando presencia de mudas; aspectos del comportamiento de ninfas y adultos también fueron registrados. Se construyó una tabla de vida mediante la combinación de datos obtenidos en siete cohortes. La localización de posturas se realizó por disección de las plantas y a través de métodos de tinción. Esta especie es

capaz de completar todo su ciclo de vida sobre alfalfa. Se estableció que las hembras de *C. nigripectus* no atienden a su progenie. Los huevos son depositados individualmente y pocas veces se encontraron dos o tres en la misma postura, la que se encuentra principalmente en los nudos (en las axilas de las hojas) o en el entrenudo (entre la epidermis y tejidos corticales). El período de pre-oviposición es corto (seis días) y la duración promedio del estado de huevo es 20 días. El período ninfal es variable, entre 32 y 63 días, con una media de 45 días. Se reconocieron dos períodos de alta mortalidad: el primer estadío ninfal, y durante la segunda semana de vida de los adultos. La longevidad promedió 61 días, pero algunos individuos vivieron hasta 202 días; las hembras viven más que los machos. En laboratorio, la proporción de sexos es aproximadamente 1,3:1 hembras:machos. Tanto ninfas como adulto son atendidos por la hormiga *Camponotus punctulatus* Mayr. Se proporcionan también datos relativos al comportamiento y distribución de la especie.

Palabras Clave: ciclo de vida; comportamiento; enfermedad de la alfalfa; fitoplasma

Membracidae Rafinesque (Hemiptera: Cicadomorpha) is a large family that includes about 3,500 known species worldwide (McKamey 1998; Wallace & Deitz 2004; Dietrich 2008; Deitz et al. 2011), and its greatest diversity is in the Neotropical region (Wood 1993; Linares et al. 2010). They are typically phloem sap feeders and a few are considered minor pests of agriculture in subtropical regions of the world, such as *Stictocephala bisonia* Kopp & Yonke, 1977 (= *Membracis bubalus* Fabricius, 1794 (in part) (the buffalo treehopper), *Umbonia crassicornis* (Amyot & Serville) (thorn bug), *Spissistilus festinus* (Say) (three-cornered alfalfa hopper), *Aconophora compressa* Walker and *Oxyrhachis* spp. (Jordan 1952; Wood 1974; Kopp & Yonke 1977, 1979; Mead 2000; Andrade 2005; Gajalakshmi & Jayakumar 2011).

Membracidae is a poorly studied group in Argentina. Most of the contributions are on taxonomy and/or geographic distribution, and very few address biological aspects (Box 1929; Christensen 1942, 1943; Torres 1953; Hayward 1960; Sakakibara 1968; Remes Lenicov 1970, 1973a, b; Linares et al. 2010). Remes Lenicov (in press) mentions 140 species from 55 genera, 14 tribes and 7 subfamilies in Argentina; where the subfamily Smiliinae is the most diverse (63 species) including 21 species of the genus *Ceresa*. This genus is widely distributed in South America with representatives in Venezuela, Colombia, Ecuador, Perú, Bolivia, Paraguay, Brazil, Uruguay and Argentina (Remes Lenicov 1973a). Only 3 species of *Ceresa* were recognized as harmful to alfalfa crops, i.e., *C. brunnicornis* (Germ.), *C. extensa* (Fairm.) (Remes Lenicov 1973a) and recently *Ceresa nigripectus* Remes Lenicov, as a carrier of ArAWB (Argentinean alfalfa witches´ broom) phytoplasma (Meneguzzi 2009).

Alfalfa is essential for Argentinean agricultural development because it is the best source of silage for various livestock to obtain meat, milk and wool. In addition, alfalfa is extremely important for conservation and recovery of productive soils. Based on the planted area (4-5 million ha), alfalfa is the second most important crop in Argentina after soybean (Gieco et al. 2007).

Diseases affecting alfalfa are a limiting factor for the production of this crop in the world; the most reliable estimate indicate that up to 25% of the forage production is lost annually as a result of the action of disease-causing microorganisms, representing losses of US$ 250 million annually (Leath et al. 1988). In Argentina, fungal, viral and bacterial diseases, which cause variable damage, have been reported (Gieco et al. 2007). The «witches´ broom» symptom is caused by the Argentinean alfalfa witches´ broom phytoplasma (ArAWB; 16Sr VII-C), and is particularly important in the Argentinean seed production areas located mainly in central and western Argentina. Affected plants show dwarfism, shoot proliferation, severely reduced leaf size, chlorosis, general stunting, and flower abortion (Conci et al. 2005). The disease significantly reduces forage yield, seed production and there are no resistant cultivars available (Gieco et al. 2007).

Phytoplasmas are vectored by hemipterans, i.e., species of Liviidae, Psyllidae, Cixiidae and mainly by Cicadellidae (Membracoidea) (Weintraub & Beanland 2006). Although no phytoplasma transmission has yet been reported for Membracidae, considering that they tend to feed on woody hosts, it would not be surprising to find that they transmit phytoplasmas primarily found in woody plants (Wilson & Weintraub 2007). Field studies on the fauna associated with alfalfa in Argentina demonstrated that one of the most abundant and frequent Auchenorrhyncha is the treehopper *Ceresa nigripectus* Remes Lenicov 1973 (Meneguzzi 2009). Laboratory studies verified the presence of ArAWB in individuals of *C. nigripectus* by PCR technique (Meneguzzi 2009), but as yet, the ability of this species to transmit the alfalfa witches´ broom disease has not been experimentally demonstrated. *Ceresa nigripectus* has a wide distribution in northern and central Argentina and it is also present in Brazil (Remes Lenicov in press); it was collected from alfalfa, potato (*Solanum tuberosum* L.) and wheat (*Triticum aestivum* L.) and surrounding vegetation (Remes Lenicov 1973a; Remes Lenicov et

al. 2004; Meneguzzi 2009). The life cycle of this membracid was unknown until now.

Considering the abundance of *C. nigripectus* in alfalfa crops in central west Argentina, and the previous studies that demonstrated that this species is a carrier of the ArAWB phytoplasma, the purpose of this contribution is to provide data on the behavioral and biological parameters of *C. nigripectus*, such as egg-laying behavior, fertility, egg viability, duration of different developmental stages, sex ratio, and longevity.

Materials and Methods

A colony of *C. nigripectus* was established with adults collected during the summer of 2012 from alfalfa fields located in Manfredi, Córdoba province (S 31° 25' -W 64° 11', 390 m asl), Argentina. They were brought to the laboratory and placed in rearing cages into a climatic chamber under the following conditions: 27 ± 2 °C, 60-80% RH and 16:8 h L:D artificial light photoperiod. The species identity was corroborated by the original description and specimens were checked with the types hosted at the Museo de Ciencias Naturales (La Plata, Argentina).

Treehopper colonies were maintained in aluminum cages of 50 × 25 × 50 cm high containing 6 healthy potted alfalfa plants (pots of 6.3 dm^3). Every side but the base was closed by nylon mesh cloth, for aeration. The cages had 2 portals for insect manipulation, one at the front and another at the back. New potted alfalfa plants were introduced in the breeding chambers as needed.

Lab-reared *C. nigripectus* nymphs, progeny of the field-collected adults, were randomly taken from the colonies and confined in the growth chamber. When these individuals reached the adult stage, their gender was determined and couples were formed. Twenty-four couples were located individually in oviposition cages made with two 10 × 10 cm transparent plastic walls surrounded by adhesive weather-strip borders (1 cm). These cages were placed to embrace the alfalfa stem containing at least one node and leaves (using a rubber band), and so, females were allowed to oviposit on the potted alfalfa plant for 24 h. The cages were repositioned on the plant every day until the death of the females; the exposed stems were labeled and followed until newly eclosed nymphs were observed. Males were replaced when they died to ensure that all females had a male for copulation every day of their lives.

Two sets of couples were made. The first 10 were used to determine the total number of laid eggs by a single female. Thus, the sections of stems containing eggs were conserved daily and dissected in order to note their position and to count them. Some exposed stems were treated using the egg staining method proposed by Curtis (1942). The second set of 14 couples was checked daily for nymphs, and to follow their development. These later nymphs were isolated in PET (polyethylene terephthalate) cylindrical cages (20 × 6 cm diam) containing an alfalfa stem and closed with a plug of wet cotton in one side and nylon mesh cloth at the top. They were checked every day while the occurrence of molts was noted. Thus, it was possible to obtain the developmental time of each individual from the time of egg laying to its death as an adult. Summary life tables for cohorts were built by combining the observational data from 7 replicates (Rabinovich 1978; Sedlaceket al. 1986; Bellows & Van Driesche 1999); eggs were not included in this analysis because we were unable to determine the exact date of egg death. Other aspects of nymphal and adult behavior were also recorded.

A Shapiro-Wilks normality test was performed for the obtained data. When appropriate, data were analyzed using a Student *t* or Wilcoxon test for mean separation at the 0.05 level of significance. All data were analyzed with the InfoStat 2013 software (Di Rienzo 2013). Voucher specimens were kept as part of the entomological collection at the Museo de Ciencias Naturales, La Plata (treehoppers), and Inst. Fundación M. Lillo, Tucumán (ants).

Results

Egg Laying and Egg Distribution in the Plant

Females laid their eggs in the stems of alfalfa plants, mostly in 2 sites: the stem node, where the eggs were partially thrust into the axil, hidden between the buds and petioles and partially covered by the plant's pubescence; and in the internode space where the eggs were entirely thrust between the epidermis and the cortical tissues. Even though occasionally eggs were laid in groups of 2 or 3, most of the time they were found laid individually.

Life Cycle

Individuals of *C. nigripectus* are solitary. After laying their eggs, females did not tend their broods. Females had a pre-ovipositional period that lasted between 4 and 10 days (n = 10, mean 6.33 ± 2.2 [SD] days), and after the pre-ovipositional period, they laid eggs during the rest of their lives. On average each female laid eggs at the rate of 0.7 eggs per day. In an experiment using 14 females, 331 eggs were checked, and the duration of the egg stage lasted 20.56 ± 6.15 [SD] days, and ranged between 5 to 49 days.

Ceresa nigripectus has 5 nymphal instars (Fig. 1), and the duration of the entire nymphal stage was 45.39 ± 6.81 [SD] days (n = 74, range: 32-63 days) (Table 1). A wide variation was found among the individuals, some reaching full maturity a week earlier than others. The male nymph-

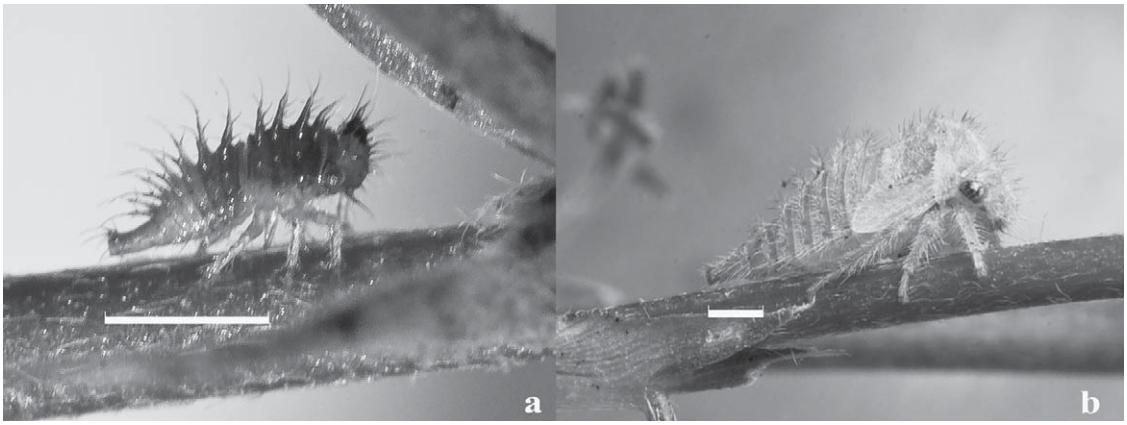

Fig. 1. Nymphs of *Ceresa nigripectus* Remes Lenicov, a) First instar. b) Fifth instar. Scale bar: 1 mm.

al stage lasted 44.44 ± 7.13 [SD] days (n = 32, range: 32-63) and the female nymphal stage lasted 46.12 ± 6.56 [SD] days (n = 42, range: 33-63). The data analysis demonstrated that there was no significant difference by gender (t test, df = 72, t = 1.05; P = 0.296). The time required for development from egg to adult emergence was nearly 66 days [mean 65.9 ± 6.5 (SD)].

After the eggs hatched, the newly emerged nymphs moved to the basal portion of the plant near the soil. Only when the nymphs reached the third instar did they begin to climb up the stem. The late instar nymphs were much more active than early instars. The progeny had a sex ratio of about 1.3:1 females:males.

In the trials carried out to determine the durations of different developmental stages, adult longevity was found to be variable with a mean of 61.35 days and a very large standard deviation (64.16 days) (Table 1); if only those specimens that lived at least 10 days (81.1% of the adults) are considered, the average longevity was 74.42 ± 64.61 [SD] days (n = 60; range = 10-202). In our comparison of the genders, we considered all the measured adults, as well as only those that lived 10 or more days; and we found that females lived substantially longer than males. The differences are statistically significant: overall average days of longevity for males was 34.1 ± 32.9 [SD] (n: 32) and for females it was 82.1 ± 74.1 [SD] (n: 42) (t test, df = 60, t = 3.74; P = 0.0004); but for those that lived 10 or more days, males lived a mean of 42.0 ± 33.1 [SD] (n = 25) days and females lived 97.5 ± 71.7 [SD] (n = 35) days (Wilcoxon test, W = 594.0, P = 0.011).

In the life expectancy (ex) curve, was possible to observe 2 critical periods characterized by an increase of the ex values (Fig. 2). The survivorship curve (lx) showed that the mortality rate was approximately constant throughout the adult stage (Fig. 2).

Other Observations

In alfalfa crops in Manfredi and Coronel Olmedo (Córdoba), all the developmental stages of the

TABLE 1. LONGEVITY AND DURATION (DAYS) OF THE NYMPHAL STAGES OF *CERESA NIGRIPECTUS* REMES LENICOV REARED ON ALFALFA PLANTS UNDER LABORATORY CONDITIONS (27 ± 2 °C, 60-80% RH AND 16:8 H L:D OF ARTIFICIAL LIGHT).

		n	Mean ± SD	range
Nymphal stage	N I	126	6.07 ± 1.57	3 - 12
	N II	122	5.87 ± 2.5	2 - 14
	N III	110	7.75 ± 2.62	3 - 17
	N IV	96	8.66 ± 2.67	3 - 21
	N V	74	16.65 ± 5.59	6 - 34
	TOTAL	74	45.39 ± 6.81	32 - 63
Adult longevity	Males	32	34.09 ± 32.91	2 - 118
	Females	42	82.12 ± 74.09	1 - 202
	TOTAL	74	61.35 ± 64.16	1 - 202

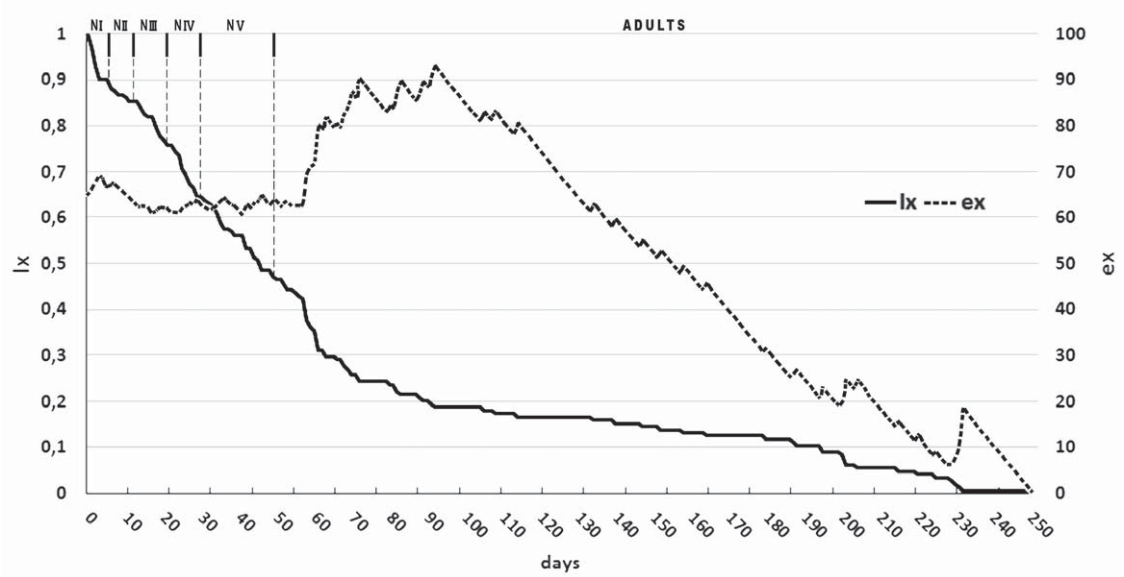

Fig. 2. Survivorship (*lx*) and life expectancy (*ex*) curves of *Ceresa nigripectus* Remes Lenicov reared on alfalfa plants at 27 ± 2 °C, 60-80% RH and 16:8 H L:D artificial light photoperiod. In addition, the mean duration of each stage is shown. (NI - NV: nymphal instars).

treehopper were tended by the ant *Camponotus punctulatus* Mayr (Hymenoptera: Formicidae), but no predatory attacks on the treehoppers were noted; the ants were observed feeding also on alfalfa nectaries. In this contribution, new distributional data of *C. nigripectus* is given for Manfredi (Córdoba province).

Discussion

Because data on the biological aspects of most Smiliinae species are very scarce at a regional level (Godoy et al. 2006), in this section we also have considered biological information gathered from other treehopper subfamilies distributed in very different geographical regions. This allowed us to gain a wider perspective on the information obtained. It is expected that the behavior of a subtropical species like *C. nigripectus* may differ from that of tropical or strictly temperate species.

Ceresa nigripectus is a solitary species, and its females do not tend their broods; Godoy et al. (2006) specified that in species of the genus *Ceresa* the adults are mostly solitary and there is no maternal care. Glass (1991) mentioned that the majority of temperate species do not guard their eggs.

Eggs of *C. nigripectus* are laid mostly at 2 locations on the plants, in the internodal space between the epidermis and the cortical tissues, and in the nodes in the axils of the leaves partially embedded in the tissues. Most *Ceresa* species laid their eggs generally in the base of internodes (Godoy et al. 2006). Funkhouser (1917) stated that *Ceresa*'s eggs are laid mostly in the bark of the stems, in deep slits, or in the buds just under the outer bud scale, and they are not fixed with a covering of a jelly-like substance, as described by Gajalakshmi & Jayakumar (2011) for *Oxyrhachis rufescens* Walker (Hemiptera: Membracidae) on *Prosopis spicigera* L. (Fabales: Fabaceae).

Unexpectedly, *C. nigripectus* laid most of its eggs individually and rarely did we find 2 or 3 eggs in the same slit. Godoy et al. (2006) mentioned that in the genus *Ceresa* the females insert their eggs in groups of 4 to 15. Funkhouser (1917) mentioned that in most of the 7 *Ceresa* species found in the Cayuga Lake basin of New York state, eggs are laid in groups from 4 to 8. Linares et al. (2010) described that the membracid *Guayaquila projecta* (Funkhouser) deposits its eggs on *Bougainvillea glabra* Choisy (Caryophyllales: Nyctaginaceae) in masses with a range of 24-72 eggs, and Snow & Dhileepan (2008) asserted that the eggs of the membracid *Aconophora compressa* Walker were deposited in batches.

We were not successful in determining the total number of eggs laid by a single female, or in building an oviposition curve because the females used in the assay died before 21 days. This parameter must be carefully considered, based on the results about the longevity of this species. Supplementary studies will be done in order to determine fecundity and fertility of the species. Godoy et al. (2006) mentioned that in *Spissistilus* spp. females can lay more than 200 eggs.

The pre-ovipositional period in *C. nigripectus* is relatively short (6 days average), compared to

20 days for the lantana treehopper *Aconophora compressa* (Membracinae: Aconophorini) (Snow & Dhileepan 2008).

In *C. nigripectus* the duration of the egg stage rounded off to a mean of 20 days, but some eggs hatched after 49 days. The length of this stage is highly variable in most of the membracids species, and it could range from 6 to 33 days, as recorded by Gajalakshmi & Jayakumar (2011) for *Oxyrhachis rufescens* Walker (mean of 5.7 days), 8-10 days for *Spissistilus festinus* (Say) (Jordan 1952), or 33 days for *G. projecta* (Linares et al. 2010).

The duration of the entire nymphal stage of *C. nigripectus* ranged between 32 to 63 days with a mean of 45 days. Funkhouser (1917) stated that the nymphal development is variable in *Ceresa borealis* Fairmaire; various individuals from the same egg cluster do not develop uniformly, some reaching full maturity a week earlier than others. Nymphal development times for other treehoppers include: a mean of 21.2 days for *S. festinus* (Jordan 1952), 26.2 days for *O. rufescens* (Gajalakshmi & Jayakumar 2011), 40 days for *G. projecta* (Linares et al. 2010), 49 days for *C. borealis*, 5 weeks for *Telamona ampelopsidis* Harris, or 6 weeks for *S. bisonia*. Funkhouser (1917) states that the life cycle of *Stictocephala diceros* (Say) is long because the fifth nymphal instar is much longer than the preceding ones; similarly, this phenomenon was observed in *C. nigripectus* (Table 1).

The death rate of *C. nigripectus* was fairly high in the first instar, as had been seen in *S. festinus* (Jordan 1952). The life expectancy curve (Fig. 1), showed 2 critical periods: one during the first 3 days of life of the nymphs, and the other - the most remarkable because of the increase of the *ex* values (Rabinovich 1978) - during the second week after the individuals reach the adult stage. These critical periods match those observed in the laboratory rearing. The survivorship curve (*lx*) resembles the theoretical type III curve, characterized by a constant ratio of deaths of individuals per unit of time.

Bibliographical data regarding *C. nigripectus* revealed that is a generalist species, affecting both dicots and monocots (Remes Lenicov 1973a; Remes Lenicov et al. 2004; Meneguzzi 2009). This is not surprising taking into consideration that other species of Ceresini also have a wide range of host plants (Godoy et al. 2006).

Funkhouser (1917) indicated that in other treehopper species, like *S. bisonia*, *Stictocephala taurina* (Fitch), and *Tortistilus inermis* (Fabr.), the first or second instars leave the tree on which the eggs hatched and migrate to succulent annual weeds, and subsequently the females that have matured return to the tree only to oviposit. In the case of *C. nigripectus*, we observed that the complete life cycle accomplished on the same host, perhaps because alfalfa can act as a woody or herbaceous plant depending on the crop's phenology. Godoy et al. (2006) cited that *Stictolobus minutus* (Funkhouser) (Ceresini) used *Taxodium distichum* (Cupressaceae) as unique host plant.

Our study demonstrated that *C. nigripectus* is a long-lived species. The longevity in adults had a mean of 61.35 days but it is highly variable (reaching up to 202 days) as can be seen from the high standard deviation obtained. This mean is consistent with the maximum longevity stated by Linares (2010) for an adult of *G. projecta* (Aconophorini). Females of the Ceresini *Spissistilus* sp. can survive nearly 40 days (Godoy et al. 2006). As was mentioned before, during the adult stage those *Ceresa* individuals that survived the critical second week, considerably increased their life expectancy. Another aspect to take into account is the fact that the mean female life span is significantly longer than the male's life span. The knowledge of field-biology of this species is still incomplete; so, we are unable to infer the biological significance of these results, e.g., like voltinism.

The progeny of *C. nigripectus* had a sex ratio of about 1.3:1 females: males. Although in all following cases there were more females than males, our result differs considerably from that of *Telamona unicolor* Fitch (Smiliini) where the males are much less numerous than the females (Funkhouser 1917). Gajalakshmi & Jayakumar (2011) recorded for *O. rufescens* (Centrotinae), on *P. spicigera* as host plant, a sex ratio of 3.5:1 and with a maximum ratio of 5.7:1 recorded during September. Conversely, for *S. festinus* Jordan (1952) recorded more males than females with a ratio of 1:1.5.

The behavior of *C. nigripectus* is very similar to the behavior of *S. festinus*, as noted by Jordan (1952). Nymphs usually remain on alfalfa fairly close to the ground, although some of them are occasionally found feeding 5 or 10 cm up the plant. As long as adequate food is available, nymphs are quite sedentary, but will search out other plants if the necessity arises. The adults have been observed resting and feeding on nearly all portions of the plant, and they move about much more readily and easily than the nymphs, which, like all treehoppers nymphs, are unable even to jump.

Like other membracids species, nymphs and adults of *C. nigripectus* were attended by ants (Funkhouser 1917; Jordan 1952; Strumpel 1972; Godoy et al. 2006).

This is the first contribution about the biology of *Ceresa nigripectus*, a widely distributed species in Argentina and one of the most abundant and frequent Auchenorrhyncha inhabiting alfalfa crops. Since this treehopper acts as a carrier of the Argentinean alfalfa witches´ broom phytoplasma (ArAWB), further research is needed to gain more insight into the role of this treehopper

in the epidemiology of the disease, and to enhance knowledge of the biology of the species.

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