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RESEARCH ARTICLE

Reproductive and brood-rearing strategies in *Alchisme grossa* (Hemiptera: Membracidae): genetic analyses of kinship relationships

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Abstract

Alchisme grossa is a treehopper species showing maternal care until at least the third nymphal instar. A secondary female treehopper has frequently been observed near a family (primary female guarding its egg clutch). Intraspecific brood parasitism, communal breeding or alloparental care may be suggested as possible mechanisms to explain secondary female presence. To distinguish between these phenomena, we performed relatedness analyses of genetic samples of groups including one *A. grossa* primary female, a secondary female and the associated offspring using polymorphic microsatellites. Furthermore, we characterized the behavioral interaction between both females during maternal care and the reproductive strategy (monandry or polyandry) of *A. grossa* females by estimating the number of male parents. We observed the presence of secondary females in 35.9% of monitored families. The behaviors characterized suggest the occurrence of brood parasitism in the interaction between both females. Nevertheless, all offspring within a family were descendants only of the primary female and a single male, thus showing that *A. grossa* females are monandrous. The results, taken together with data on the reproductive biology reported for other treehoppers, are consistent with the occurrence of brood parasitism in *A. grossa.*

Keywords Alloparental care · Communal breeding · Intraspecific brood parasitism · Reproductive strategy · Monandry · Microsatellite

Introduction

Parental care requires investment in time and energy by one or both parents (Wong et al. 2013) and involves costs that can affect future reproductive events (Zink 2003a) and the survivorship of parents (Suzuki et al. 2005). These costs may be compensated if offspring survival increases as a consequence of parental care, thereby increasing the indirect fitness of one or both parents (Wong et al. 2013; Hamilton 1964; Alonzo and Klug 2012).

In insects, parental care is most frequently provided by the female (i.e., maternal care; Wong et al. 2013) and consists mainly indirect attendance and guarding behaviors, food provisioning, food facilitation, or protection of resources required by the offspring (Wong et al. 2013; Tallamy and Wood 1986; Royle et al. 2012). In treehoppers (Hemiptera: Membracidae), maternal care has been shown to be essential for egg-hatching as well as for feeding and survival of first nymphal instars, as is the case in *Umbonia crassicornis* (Wood 1974), *Platycotis vittata* (Wood 1976), *Guayaquila compressa* (Wood 1978), *Publilia reticulata* (Briwston 1983), *Umbonia ataliba* (Masters 1989), *Entylia*

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bactriana (Olmstead and Wood 1990), *Publilia concava* (Zink 2003a, b), *Ennya chrysura* (Miranda 2016), *Alchisme grossa* (Torrico-Bazoberry et al. 2014) and *Ennya maculicornis* (Caceres-Sanchez et al. 2017).

In general, semelparous species provide extended parental care to their offspring (i.e., care is provided for a long period of time, usually up to juvenile individuals), while in iteroparous species parental care is less extended since parents distribute their reproductive investment (including parental care) along with several events (Tallamy and Brown 1999; Trumbo 2013). Species belonging to the treehopper tribe Hoplophorionini exhibit extended maternal care (i.e., maternal care is observed through the egg and almost all nymphal stages) and have been suggested in general to be semelparous (Wood 1974; Godoy et al. 2006; Lin 2006), except for *A. grossa*, which has been proposed as a moderately iteroparous species (Torrico-Bazoberry et al. 2014). *A. grossa* exhibits maternal care until at least the third nymphal instar including feeding facilitation towards newly hatched nymphs (Torrico-Bazoberry et al. 2016).

It has been frequently observed in *A. grossa* that another female (i.e., a secondary female) approaches a primary female guarding its egg clutch (i.e., a family) and stays near this family for some days (Torrico-Bazoberry et al. 2014). This presence of secondary females may be potentially explained by three general mechanisms. First, alloparental care, a phenomenon that involves the provision of care to offspring of other individuals, implies a type of interaction in which the individual providing care is not a parent of any of the offspring (Hamilton 1964; Wisenden 1999; Loeb et al. 2000; Eggert 2014; Zink and Lyon 2016). Second, communal breeding is a type of cooperative brood care involving two or more parents; if females that take care of the progeny are closely related, they gain inclusive fitness by caring for each other's offspring (Trumbo 1992; Eggert and Sakaluk 2000; Zink 2003a, b, Zink 2005; Wong et al. 2013). Third, intraspecific brood parasitism occurs when a female lays eggs on or near the egg clutch of a primary female, thus taking advantage of the primary female's maternal care and minimizing or avoiding her own costs of maternal care

(Zink 2000, 2003a; Tallamy 1985; Field 1992; Stovicek et al. 2013). The likelihood of occurrence of brood parasitism may be associated to the degree of relatedness between parasite and host females (Hamilton 1964; Eberhard 1975, 1986; Emlen and Wrege 1986; Zink 2000, 2003a; Zink and Lyon 2016; Andersson et al. 2019). Also, brood parasitism is more likely to occur if a primary female does not defend her egg clutch successfully (Zink 2000). A summary of the attributes associated with the occurrence of the three different mechanisms to explain the presence of secondary females is presented in Table 1.

To identify the mechanism of interaction between primary and secondary females of *A. grossa*, natural history data as well as behavioral and genetic data are needed. Hence, we focused on analyzing behaviors of primary and secondary females, for example, how long the secondary female remains near the primary female and her egg clutch, the tolerance or aggressive behaviors displayed between both females, and the hatching success of eggs in the presence or absence of a secondary female. We also coupled behavioral data with genetic relatedness analyses of *A. grossa* primary females, their offspring and their associated secondary female using polymorphic microsatellites developed for this species. To explore other variables potentially associated with female behaviors observed, we described the reproductive strategy (monandry or polyandry) of *A. grossa* females by estimating the number of male parents.

Materials and methods

Sampling site

This study was performed at the Santa Isabel locality (17°11′49″ S, 65°50′43″ W, 2300 m.a.s.l.) in the Yungas biogeographical region of Cochabamba, Bolivia from March to April 2018. Treehoppers were observed on two host plants: *Brugmansia suaveolens* and *Solanum ursinum* (both Solanaceae). In their habitat, *A. grossa* shows nymphal and adult aggregations on their host plant, where

Table 1 Attributes associated with the occurrence of three different mechanisms to explain the presence of secondary females in *A. grossa* families

	Alloparental care (Hamilton 1964; Wisenden 1999; Loeb et al. 2000; Eggert 2014; Zink and Lyon 2016)	Brood parasitism (Zink 2000, 2003a; Tallamy 1985; Field 1992; Stovicek et al. 2013)	Communal breeding (Trumbo 1992; Eggert and Sakaluk 2000; Zink 2003a, b, 2005; Wong et al. 2013)
Aggression toward secondary female	No	Yes	No
Oviposition by secondary female	No	Yes	Yes
Parental care by secondary female	Yes	No	Yes
Kinship between females	Yes	No	Yes

females perform a continuous maternal care at least until the end of third instar (Torrico-Bazoberry et al. 2016). Maternal care is performed through behaviors such as egg guarding, feeding facilitation for nymphs in initial instars and active defense against predators (Torrico-Bazoberry et al. 2014).

One hundred and ninety-eight primary ovipositing females were marked on their pronota with a Sharpie marker following Torrico-Bazoberry et al. (2014). A female and her eggs or brood are referred to as a family. When another female (i.e., a secondary female) was observed near a family she was also marked. Different codes were used for each primary and secondary female, and different colors were used to distinguish between families. In addition, we defined two periods during the egg stage: the oviposition period (ca. 10 days) during which females lay their eggs in a clutch, and the egg development period (ca. 21 days) during which females guard their eggs continuously by remaining on top of the egg clutch until they hatch. Visits by secondary females were observed in 71 families. Observations were initiated at the moment that a secondary female was observed accompanying a family; they were performed twice daily (morning and afternoon) in 45-min observation events during all the time that the secondary female remained with the family (i.e., each family and secondary female were observed for 90 min daily). The number of secondary females present on each of the previously described periods were compared using a chi-square test. Presence or absence of primary and secondary females was recorded and the effect of the presence of a secondary female on the hatching of egg clutches determined using a Fisher's exact test. Further, occurrence or non-occurrence of the following behaviors was registered: egg clutch defense (i.e., aggressive behaviors) by the primary female and oviposition and egg care by the primary and secondary females.

Secondary females were observed to remain nearby (within an ~ 3.6 cm radius of the family) for 1.9 ± 1.2 days. Secondary females were collected for genetic analysis three days after they were marked. This time-lapse gave them a chance to oviposit as has been seen in *P. dispar* (Eberhard 1986), while diminishing the risk that they flew away before collection, particularly given that the rate of a recapture of secondary females was only 30.9%. This led to the analysis of 10 families which showed a constant presence of secondary females. Observations in these families continued until nymphs reached the third instar; thereafter, primary females and their offspring were collected. Specimens collected were preserved in 70% ethanol and stored at −20 ºC, until performing genetic analyses. In the remaining 61 families, observations were continued until the third instar of the nymphs.

Genetic analyses

The ten families studied contained from 22 to 55 nymphs. All nymphs were used in the analysis when there were fewer than 30. When there were more, 30 nymphs were randomly chosen. Thus, a mean of 79.7% (54.5–100%) of offspring in each family was analyzed. DNA was obtained from the primary female, the secondary female (the first secondary female to approach the family was analyzed) and the nymphs using the salt extraction protocol described by Aljanabi and Martinez (1997). Each sample was diluted to a DNA concentration of 60 ng/µl for genetic analyses. Eight microsatellites were amplified from each sample as described in Online Appendix 1.

Polymerase chain reaction (PCR) was performed on mixtures (12 µL) containing 2 µL (100 ng) of template DNA, 0.2 µL of forward primer (0.25 µM), 0.5 µL of reverse primer (0.25 µM), 2.4 µL of dNTP (100 µM dNTP) (Invitrogen), 1.3 µL of $10 \times$ PCR buffer (0.96×), 0.5 µL of $MgCl_2$ (2 mM), 0.12 µL of *Taq* polymerase (0.5 U) (Invitrogen), and 3.88 µL of molecular water. Cycling conditions consisted of an initial denaturing step of 4 min at 95 °C, followed by 10 touchdown cycles consisting of 30 s at 95 °C, 1 min at 68 °C decreased by 1 °C in each cycle and 1 min at 72 °C, followed by 25 annealing cycles consisting in 30 s at 95 °C, 1 min at 58 °C and 1 min at 72 °C, and a final elongation step at 72 °C for 5 min. PCR products were sent to the sequencing service at Pontificia Universidad Católica de Chile which uses an Applied Biosystems automatic sequencer. Allelic data were scored using the GeneMarker software (SoftGenetics).

Statistical power to detect multiple paternity in broods

Since we did not genotype the complete clutch of each female, statistical power to detect multiple paternity was estimated using the power of multiple paternity described by Veliz et al. (2017). This method estimates the probability of collecting at least one sample with nymphs from several progenitors (multiple paternity) among several samples, each from a different clutch. To do this, we used the following variables and values: (i) the mean number of nymphs sampled in each clutch:23; (ii) the number of clutches sampled: 10; (iii) the proportion of broods with a single female: 0, 0.5, or 0.75; (iv) the proportion of broods with multiple females: 1.0, 0.5, or 0.25; (v) the estimated total number of nymphs per brood: 56; (vi) the proportion of nymphs from the primary female in a brood composed by nymphs coming from several females: 0.5, 0.75, or 0.90; and (vii) the proportion of nymphs from a secondary female in a brood composed of nymphs coming from several females: 0.5, 0.25, or 0.10. The values for (i) and (ii) were the mean number of nymphs genotyped and the number of broods sampled in this study,

respectively; the value for (v) was the highest number of nymphs per clutch from our samples. Information for (iii), (iv), (vi) and (vii) are not available for treehopper species; hence, we used a series of simulations with different values to obtain different values of probability from different proportions of primary and secondary females and from different proportions of nymphs coming from the primary or the secondary female.

Relatedness analyses

The allele sequence was analyzed using the software packages PASOS, IDENTIX, GERUD and ML-Relate. The genetic relationship between nymphs and each of the two females (primary and secondary) was estimated using PASOS (Duchesne et al. 2005) a parental allocation software based on parental pair likelihood with a subsequent filtering procedure. In the present case, both females were used as possible parents and the nymphs as the progeny to allocate. Additionally, the coefficient of relatedness, I_{xy} (Belkhir et al. 2002), among nymphs of each family, between primary and secondary females, and between primary females and males for each family were estimated using IDENTIX (Belkhir et al. 2002). Differences were assessed using a Kruskal–Wallis test in SigmaPlot 14.0. The permutation test implemented in the IDENTIX software was used to detect the significance of relatedness within each brood studied. To detect the number of fathers involved in the parenthood of the group of nymphs related to each primary female, a reconstruction of parental genotype was performed with GERUD (Jones 2001). This software determines the minimum number of fathers in a group of progeny when the maternal genotype is known.

Finally, to determine the relatedness among the primary female, the secondary female and the reconstructed genotype of the potential fathers, relatedness values were estimated by using ML-relate (Kalinovwski et al. 2006), which uses maximum likelihood to estimate relatedness and relationship for pairs of individuals (K_{xy}).

Results

Ecological and behavioral observations

Seventy-one of the 198 monitored families (35.8%) received at least one secondary female visit. Among the 71 families in which secondary females were observed, 48 families received the visit of a single secondary female (67.6%), 14 families received the visits of two different secondary females at different times (19.7%), and 9 families received the visits of three or more different secondary females (i.e., 3–5) at different times (12.7%). Forty-eight secondary females were present during the oviposition period of the primary female, 15 during the egg stage, and only 8 of the 71 secondary females were observed near a family after the egg clutch hatched. The number of visits of secondary females was compared between development periods; the oviposition period was the most visited one ($X^2 = 47.67$, $df = 2$, $p < 0.001$) (Fig. 1). Secondary females maintained their positions near the families at an average distance of 3.6 cm, for around 1.9 ± 1.2 days.

Of the 198 observed egg clutches, 106 (53.5%) did not hatch. Hatching was prevented by herbivory (9 cases, 8.5%), predation (12 cases, 11.3%), anthropogenic actions (29 cases, 27.4%) and abandonment of egg clutches by the primary female (56 cases, 52.8%). In five of these 56 families, the primary female was observed near another family after abandoning its original egg clutch. Abandonment of the eggs by the primary female led to their desiccation if the female did not return within 1 day. In another four cases, the primary female started a new egg clutch that did not hatch. Thus, among clutches that hatched, 38 received visits by a secondary female and 54 did not. Among clutches that did not hatch, 33 received visits by a secondary female and 73 did not. Hatching was not affected by the presence of a secondary female (Fisher's Exact test: $df = 1$, $P > 0.05$). Six marked secondary females were observed starting their own egg clutch, but these did not hatch.

We observed eight instances of aggressive behavior from primary females toward secondary females, in which the primary female remained above her egg clutch, started fluttering her wings and generating a buzzing sound, and kicking strongly against the secondary female, occasionally hitting it. In five of these cases the secondary female moved away for a time (i.e., at least 20 cm away from
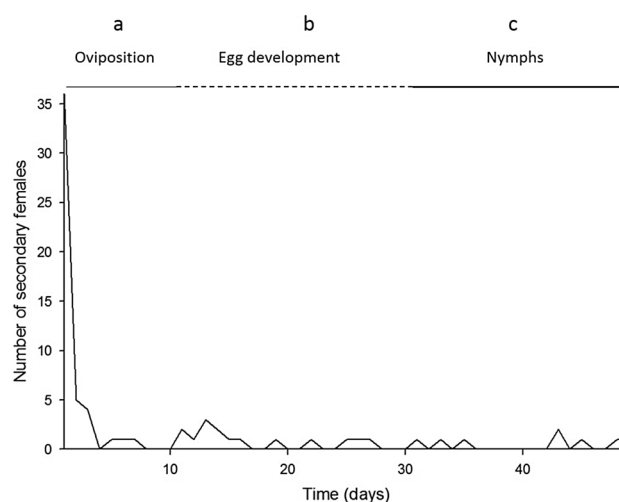

Fig. 1 Number of secondary females observed per developmental period in *A. grossa* families. Letters indicate statistical differences between periods ($p < 0.001$)

the family) but after some time she returned to her previous position (i.e., < 5 cm from the clutch). This suggests that a primary female can aggressively repel a secondary female but cannot fully prevent her from returning. Further, 10 secondary females were observed visiting two or even three different families within the oviposition period (i.e., while primary females were ovipositing their egg clutches). Another four secondary females performed two visits to the same family with a mean gap of three days between visits. Finally, another six secondary females, after being present for some time next to a family, oviposited their own egg-masses elsewhere on the same plant but away from the primary female's clutch.

Statistical power and relatedness analyses

An average of 39.5 nymphs (22–56) hatched from the egg clutches of the 10 families collected for genetic analyses. A mean of 28.3 nymphs per family (22–30, amounting to 71.6% of nymphs from each family, with a range of 66–100%) was subjected to PCR and 66.3% of these samples were successfully amplified. The statistical power test to detect a multiple brood coming from different progenitors reached values of power of multiple paternity = 0.99 in all cases when > 75% of broods have single maternity and > 90% of nymphs come from the primary female. Thus, our analysis indicated that the statistical power to detect multiple maternity is high, even with the sampling design that does not consider all the nymphs in each brood of *A. grossa*.

The parental allocation analyses using PASOS showed that all the offspring within the family were closely related to the primary female in all 10 families while none of the offspring was related to the secondary female. The multilocus paternity analyses using GERUD indicated in all cases the presence of only one father for the offspring. The relatedness analysis using IDENTIX indicated that nymphs in each family were more relationship with primary than secondary females (Fig. 2). Relatedness between primary and secondary females and between primary females and males was similar and significantly lower than relatedness between nymphs (Kruskal–Wallis test followed by Tukey test: $H = 19.400$, $df = 2$, $P < 0.001$; Fig. 3). The permutation test performed to assess the level of relatedness within each brood revealed the presence of highly related individuals in all broods analyzed ($P < 0.001$). Additionally, analysis using ML-relate indicated that primary and secondary females were neither full siblings nor half siblings (K_{xy} ranging from 0.00 to 0.04, indicating unrelated relationships), and that primary females and the reconstructed male genotype were neither full siblings nor half siblings (K_{xy} ranging from 0.00 to 0.04, indicating unrelated relationships).
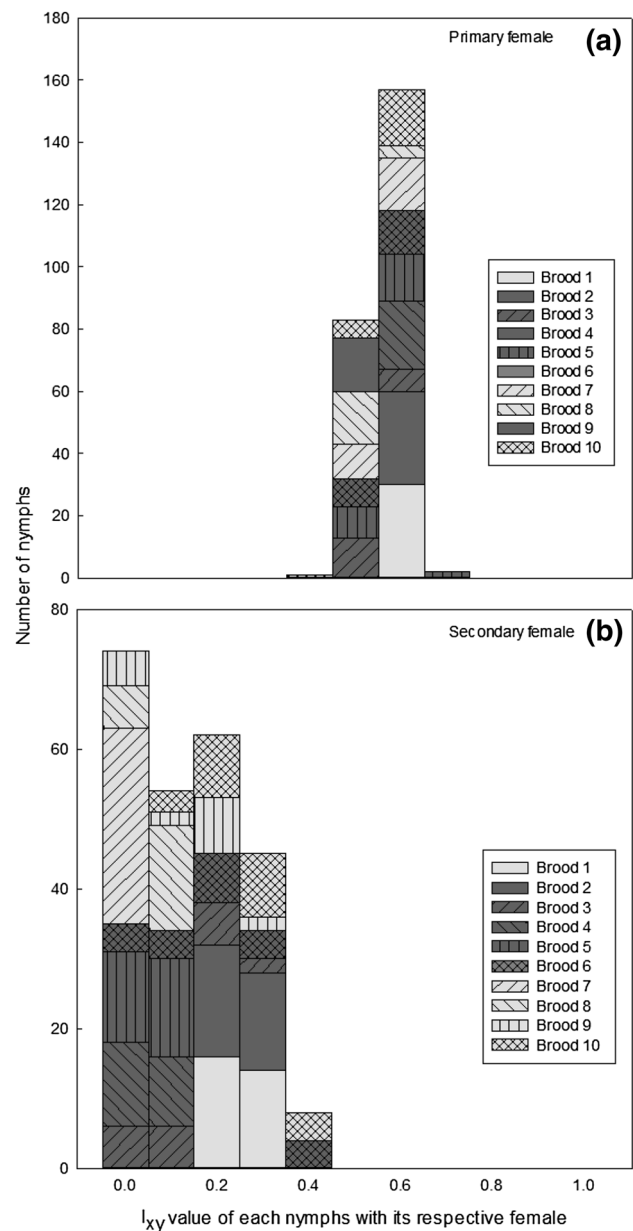
Fig. 2 Relatedness (I_{xy} values) for each nymph with its respective (**a**) primary and (**b**) secondary female. Nymphs from each brood are shown with different texture patterns

Discussion

In insects, brood parasitism has been shown to occur under three circumstances. First, it can occur when a female has failed on a first oviposition event (Zink 2003a; Field 1992; Stovicek et al. 2013; Yom-Tov 1980; Tallamy 2005). For example, the membracids *P. dispar* and *P. concava* (Membracidae: Polyglyptini) are species capable of ovipositing egg clutches in more than one event; when they fail to oviposit, some females will take the option of brood parasitism (Eberhard 1986; Wood 1993; Zink 2003a). Second, brood
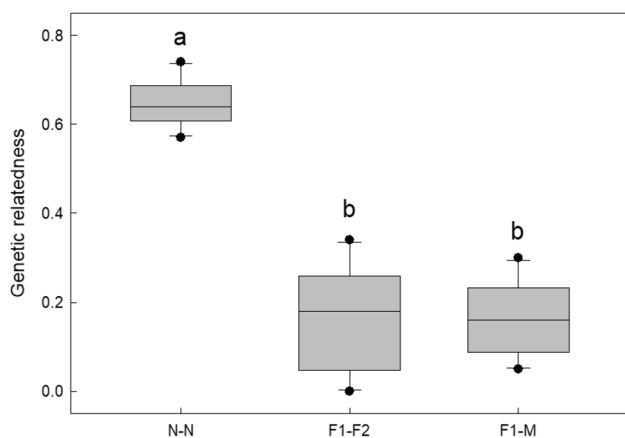
Fig. 3 Coefficient of relatedness between different groups of treehoppers, calculated using IDENTIX. *N* nymph, *F1* primary female, *F2* secondary female, *M* male. The male genotype was reconstructed using GERUD. Different letters above bars indicate significant differences ($p < 0.05$)

parasitism occurs when a female has a high fecundity and oviposition rate and oviposition is distributed among several eggs clutches (Eberhard 1986; Olmeast and Wood 1990; Alhund and Andersson 2001). For example, *P. concava* has high fecundity (Zink 2003a), and *Polyglypta dispar* has a spermatheca where it can store sperm and ovarioles and even some eggs at different developmental stages (Eberhard 1986); in both species, these features have been claimed to lead to brood parasitism. Third, brood parasitism occurs when a high number of egg clutches are present, in association with a limited resource. For example, a shortage of nesting substrate has been claimed to increase the chance of appearance of parasitic females in *P. concava* (Zink 2003a). In this case, the presence of limited resources significantly increased the time that a female needs to find an adequate substrate to oviposit, in which case some females left their eggs among the egg clutches of other females thus showing brood parasitism.

Since *A. grossa* shows moderately iteroparous reproductive behavior and produces large clutches (Torrico-Bazoberry 2014), brood parasitism seemed the most likely explanation for the presence of secondary females. However, all nymphs genotyped in each of the 10 families collected for genetic analyses corresponded to the brood of a single female and we did not observe secondary females ovipositing on the egg clutches of primary females; these results do not support the hypotheses of brood parasitism nor communal breeding. Notwithstanding, brood parasitism cannot be completely ruled out since primary females showed extended care and intolerance to the approach of secondary females through aggressive behaviors towards them (it should be noted that observations were not continuous and comprised less than 6% of the total duration of the phenomenon studied), a phenomenon previously described in treehopper species showing brood parasitism (Eberhard 1986; Zink 2003a). Moroever, the presence of secondary females was significantly higher during the oviposition period, also suggesting a potential parasitic behaviour.

An alternative explanation is that secondary females provide some sort of alloparental care. Alloparental care is more likely if primary and secondary females are closely related, in which case the secondary female may gain inclusive fitness through the care of another female's offspring. In the present study, primary and secondary females were not related and primary females showed aggressive behaviors towards secondary females, thus suggesting a lack of tolerance and cooperation between females (Eberhard 1986; Zink 2003a). However, other positive female–female interactions could have been occurring which were not formally assessed; these interactions may have generated benefits consisting in an increase in the overall fitness of the interacting treehopper individuals through, for example the reduction of predation and feeding facilitation (McEvoy 1979; Stamp 1981; Cocroft 2001; Morales et al. 2008). In line with this interpretation, both females were shown to remain at a short distance from each other, thus constituting a better defense against parasites and predators.

The reproductive biology of most treehopper species is unknown (Lin 2006). The genetic analyses performed pointed to a single possible father of all the families studied. Thus, females of *A. grossa* could have oviposited after copulation with a single male, as has been observed in *Umbonia crassicornis*, another Hoplophorionini treehopper (Wood 1974). On the other hand, the possibility that females of *A. grossa* may mate more than once, store the sperm and then use the sperm of just a single male to fertilize the eggs, as has been observed in *P. vittata* (Wood et al. 1984), cannot be discarded. While it seems likely that monoandry is the reproductive strategy of *U. crassicornis* and *A. grossa*, polyandry has been suggested in other species such as *Platycotis vittata* (Wood et al. 1984) and *Ennya maculicornis* (Cossio-Rodriguez et al. 2019). The reproductive strategy of males (monogyny or polygyny) remains unknown; further investigations should be pursued to fully understand the reproductive strategy of *A. grossa*.

Lastly, the results presented indicate that in *A. grossa* the primary female shows a low level of relatedness to the male. Adults show low dispersal; females remain to a large extent on the same plant where they were born, while males may move to other plants within a 20-m radius from their maternal plant (C. F. Pinto, pers. obs.). These results suggest the occurrence of inbreeding avoidance which may reduce the effect of inbreeding depression in the progeny (Kristensen et al. 2010; Aguilera-Olivares et al. 2015). This finding is in line with other treehopper species in which it has been suggested that individuals avoid mating with siblings (Wood

and Dowell 1985). However, mating between siblings has been observed in some treehoppers (Eberhard 1986; Masters 1989; Wood 1993). The factors involved and the cost–benefit considerations in mating with sibling or non-sibling individuals in treehoppers have not been adequately addressed.

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