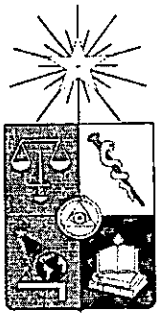

PATRÓN DE USO DE HOSPEDEROS POR *ALCHISME GROSSA* (HEMIPTERA: MEMBRACIDAE): MECANISMOS CONDUCTUALES Y CONSECUENCIAS ECOLÓGICAS Y GENÉTICAS

Tesis

Entregada a la Universidad de Chile

En cumplimiento parcial de los requisitos para optar al grado de

Doctor en Ciencias con mención en Ecología y Biología Evolutiva

Facultad de Ciencias

Carlos Fernando Pinto Navia

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Santiago, Octubre 2015

INFORME DE APROBACIÓN

Se informa a la Escuela de Postgrado de la Facultad de Ciencias que la Tesis de Doctorado presentada por el candidato:

Carlos Fernando Pinto Navia

Ha sido aprobada por la comisión de evaluación de tesis como requisito para optar al Grado de Doctor en Ciencias con mención en Ecología y Biología Evolutiva, en el examen de Defensa Privada de Tesis rendido el día 7 de Octubre de 2015

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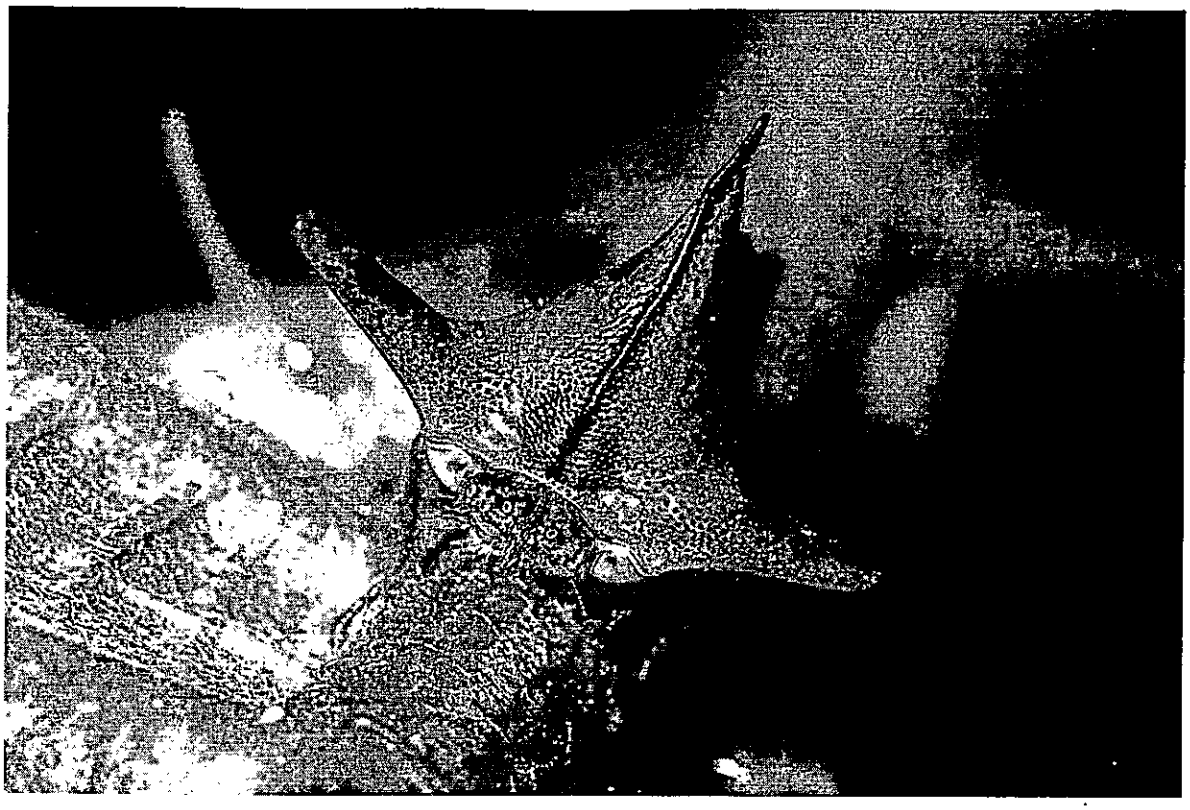

A todas mis madres y padres, por llevarme de la mano

y seguirme guiando y cuidando en esta aventura

laboratorio y todos sus miembros han sido para mi también una familia y una escuela en el día a día. Aunque llevo más de 10 años estudiando insectos, poco puedo decir respecto a denominarme entomólogo o no; lo que sí afirmo categóricamente es que aún logro asombrarme en el día a día al observar estos animales apasionantemente interesantes.

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LISTA DE ABREVIATURAS

AG = *Alchisme grossa*

BS = *Brugmansia suaveolens*

SU = *Solanum ursinum*

m = metro

km = kilometro

mL = mililitro

MCMC = Monte Carlos Markov Chain

µL = microlitro

MDT = Maximum distance traversed

MAR = Mean activity radius

MCP = Minimum convex polygon

DIC = Deviance information criterion

GLM = General linear models

TA = Tropanic alkaloid

F_{ST} = Diferenciación genética

GC-MS = Cromatografía de gases acoplada a espectrómetro de masas (por sus siglas en inglés)

ADN = Acido desoxirribonucleico

min = Minuto

PCR = Reacción en cadena de la polimerasa (por sus siglas en inglés)

SE	=	Error estándar
N	=	Número de individuos
IR	=	Indice de retención

RESUMEN

Los insectos fitófagos y las plantas con las que interactúan constituyen una importante proporción de la macrodiversidad natural, donde la amplitud de la dieta de un insecto está compuesta usualmente por un número limitado de especies de plantas, mostrando distintos niveles de especialización. Cuando un insecto es observado utilizando más de un hospedero en simpatría, se sugiere la existencia de al menos dos potenciales escenarios: a) alternancia de hospederos, donde una población de insectos utiliza distintos hospederos al mismo tiempo y b) hospederos alternativos, donde distintos grupos de la misma especie de insecto utilizan diferencialmente los hospederos. En este sentido, surge la pregunta general de cómo puede evolucionar la especialización ecológica cuando un insecto subsiste en más de un hospedero en simpatría, bajo un escenario de uso de hospederos alternativos, lo cual puede o no conducir a diversificación vía especialización ecológica.. Los membrácidos (Hemiptera), son insectos fitófagos especializados en pocos grupos de plantas, aspecto que se ha relacionado con la química de la interacción. Específicamente, *Alchisme grossa* (Hoplophorionini) es un membrácido subsocial que utiliza dos especies de solanáceas en simpatría en los yungas de Bolivia – *Brugmansia suaveolens* y *Solanum ursinum* -, este insecto muestra un complejo cuidado maternal, donde las ninfas se encuentran limitadas a su planta de nacimiento durante todo su desarrollo. Además, estudios previos en esta localidad demostraron principalmente un uso de hospederos alternativos por este insecto. El

presente trabajo se focalizó en estudiar mecanismos y consecuencias asociadas al patrón de uso de hospederos por *A. grossa*. Para cumplir este objetivo se procedió comparar entre individuos de *A. grossa* desarrollados en cada hospedero: 1) el nivel de fidelidad alimenticia y de la selección directa o indirecta efectuada por *A. grossa* durante la dispersión de individuos sobre ambos hospederos, 2) el desempeño a nivel individual y poblacional (demografía), además de caracterizar cada hospedero como sustrato de desarrollo, 3) la interacción química entre la especie hospedera y *A. grossa* y 4) el nivel de estructuración genético poblacional de *A. grossa* que se desarrollan en ambos hospederos. Los resultados muestran una tendencia de *A. grossa* hacia preferir alimentarse de su hospedero de desarrollo y a la vez confirma un patrón de selección directa, donde los individuos se dispersan mayoritariamente solo en la especie de desarrollo. Por otro lado, el desempeño y las tendencias demográficas son similares entre hospederos, a pesar de que cada especie de planta constituye un ambiente muy distinto. Se demostró que *A. grossa* secuestra tres tipos de alcaloides de *B. Suaveolens.,* Además, no se evidenció estructuración genética entre grupos subsistiendo en cada hospedero alternativo. Los resultados son discutidos en relación a las implicancias de la filopatría caracterizada para *A. grossa*, además de los potenciales factores equilibrando el desempeño de los insectos en ambos hospederos, asociando los mismos con la química de la interacción insecto-planta y la ausencia de estructuración genético-poblacional.

ABSTRACT

Phytophagous insects and plants with which they interact constitute a significant proportion of natural diversity, where insect diet breadth is usually composed of a limited number of plant species, showing different levels of specialization. When an insect is observed using more than one sympatric host, there are at least two suggested scenarios: a) host alternation, where an insect population uses different hosts at the same time and b) alternative hosts, where different groups of the same insect species perform a differential use of hosts. In this sense arise the question about how ecological specialization can evolve when an insect exists in more than one sympatric host, under an scenario of alternative hosts where diversification via ecological speciation can occur or not. Treehoppers (Hemiptera) are phytophagous insects with patterns of specialization in a few groups of plants, factor that is suggested to be linked with chemical interaction. Specifically, *Alchisme grossa* (Hoplophorionini) is a subsocial membracid using two sympatric species of the Solanaceae family in the Yungas of Bolivia - *Brugmansia suaveolens* and *Solanum ursinum* - this insect shows a complex maternal care, where nymphs are limited to their birth plant throughout its development. In addition, previous studies at this location showed principally a potential pattern of alternative hosts use for this insect. This work is focused in the study of mechanisms and consequences associated with host use pattern by *A.* grossa. To meet this objective we proceed to compare between individuals of *A. grossa* developed in each host: 1) the level of

feeding fidelity and the presence of direct or indirect selection performed by *A. grossa* during dispersion of individuals on both hosts, 2) performance at individual and population level (demography), besides of characterize each host as developmental substrate, 3) patterns of chemical interaction between *A. grossa* and hosts and 4) the level of population genetic structure of *A. grossa* individuals developed on each host. Results show a trend of *A. grossa* to prefer to feed on their developmental host and also confirm a pattern of direct selection, where individuals disperse mostly only in the developmental host pant species. On the other hand, performance and demographic trends are similar between hosts, although each plant species constitute a very different environment. It was shown that *A. grossa* sequesters three types of alkaloids of *B. suaveolens.* In addition, the absence of genetic structure between groups subsisting on each alternative host was observed. Results are discussed in relation with implications of philopatry characterized for *A. grossa,* besides of potential factors balancing performance of insects in both hosts, associating themselves with the chemistry of the insect – plant interaction and the absence of population genetic structure.

1. INTRODUCCIÓN

La biodiversidad terrestre está dominada en gran parte por plantas y los insectos que las consumen, constituyendo de esta manera uno de los conductos más importantes para el flujo de energía hacia niveles tróficos superiores (Futuyma & Agrawal 2005). Las hipótesis macroevoutivas sostienen que la evolución recíproca de adaptaciones insecto – planta, acompañadas de procesos de especiación, son los que han dado lugar a la diversidad observada actualmente en la naturaleza (Futuyma & Agrawal 2005, Schoonhoven 2005). De un estimado de alrededor de dos millones de especies de insectos existentes, 500 000 aproximadamente corresponden a insectos fitófagos, en los cuales ha evolucionado la capacidad de buscar, reconocer y discriminar sus potenciales hábitats, utilizando para ello una variedad de señales sensoriales de naturaleza química, visual, acústica y táctil de su ambiente (Ehrlich & Raven 1964, Bernays & Chapman 1994, Schoonhoven *et al.* 2005). Las plantas, que se constituyen en hábitat-hospedero para este tipo de organismos, han generado a su vez defensas que limitan el éxito de los fitófagos que las consumen (p.e. barreras químicas, mecánicas). Por su parte, en la mayoría de los insectos se observa la evolución de características que les permiten superar las barreras de sus plantas hospederas a través de mecanismos sensoriales, fisiológicos y bioquímicos (Bernays & Chapman 1994, Nishida 2002, Schoonhoven *et al.* 2005, Desprès *et al.* 2007).

La amplitud de la dieta, representada por el rango de plantas hospederas de un insecto fitófago que se desarrolla en la misma planta de la que se alimenta, está impuesta por factores de naturaleza histórica, fisiológica, morfológica y ecológica (Bernays & Chapman 1994, Schoonhoven 2005). El efecto de ellos puede verse reflejado en el grado de restricción (o amplitud) alimenticia observada; en este sentido, una sorprendente característica de las interacciones insecto – planta es el alto grado de especialización alimenticia (monofagia y oligofagia) que predomina en sistemas naturales (Berenbaum 1996, Caillaud & Via 2000, Schoonhoven 2005). Los insectos fitófagos pueden tener amplitudes de dieta basadas en una, pocas o varias especies o familias de plantas hospederas (Bernays & Chapman 1994). En los casos donde se observa a una especie de insecto fitófago utilizando dos o más especies hospederas en una sola localidad geográfica, se presentan dos posibilidades: a) uso de hospederos alternativos, donde existirían poblaciones de insectos relativamente aisladas utilizando distintos hospederos, o b) alternancia de hospederos, donde una población de insectos utilizaría más de una especie hospedera con distintos patrones de alternancia en el tiempo (Agrawal 2000, Cunningham & West 2001, Schoonhoven 2005). Se ha sugerido que el uso de hospederos alternativos puede tener consecuencias en la ecología y la biología de los individuos, afectando rasgos tales como la duración del ciclo de vida, produciendo diferenciación en la producción y percepción de señales (p.e. señales involucradas en reconocimiento, apareamiento, alarma) (Nosil *et al.* 2007),

generando cambios en características que operan durante la elección de pareja y, en general, modificando los contextos sociales dependientes de cada tipo de hospedero (Cocroft *et al.* 2008, 2010, Lin & Wood 2002). Por otra parte, durante las etapas iniciales de un proceso de cambio de hospedero puede desarrollarse un patrón de fidelidad hacia el hospedero de desarrollo, el cual también puede actuar como un factor facilitador para la selección divergente operando sobre rasgos asociados al desempeño en cada hospedero y sobre la preferencia en los mismos (Wood *et al.* 1999).

El uso de un nuevo ambiente, ya sea como recurso trófico o hábitat, está mediado por modificaciones en el comportamiento de los organismos, que han evolucionado mecanismos conductuales específicos que les permiten ser eficientes en realizar la búsqueda y colonización de nuevos hábitats (Bell 1990, Bernays & Chapman 1994, Futuyma & Moreno 1998). La selección natural actúa en ese punto sobre los rasgos morfológicos o fisiológicos que estuvieran expresados en los individuos que colonizan nuevos ambientes; por lo que la conducta constituye parte importante del mecanismo a través del cual se realiza este proceso (Futuyma & Moreno 1998).

Los modelos clásicos para el estudio de los mecanismos y consecuencias de la selección de hábitat en insectos, han sido aquellos donde el insecto se desarrolla y reproduce en un solo hospedero. Estos modelos han sido fundamentalmente estudiados en insectos fitófagos y parásitos (Wiegmann *et al.* 1993, Berlocher &

Feder 2002). Por lo mismo, una pregunta importante en la actualidad es acerca de cómo puede ocurrir el desarrollo de especialización ecológica cuando hospederos nuevos y antiguos subsisten en simpatría, situación donde diversos procesos tales como la evolución de fidelidad hacia el hospedero de desarrollo, la coordinación de la fenología entre plantas e insectos y la ocurrencia de apareamiento sesgado podrían eventualmente conducir a una especiación ecológica (Futuyma & Moreno 1988, Jaenike 1990, Futuyma & Agrawal 2005, Schoonhoven 2005). Por otro lado, la ecología y evolución del patrón de uso de hospederos en insectos fitófagos podrían ser mejor entendidas si la preferencia y posterior desempeño en hospederos alternativos fueran estudiadas conjuntamente; sin embargo, en la actualidad son muy escasos los estudios de este tipo en ambientes naturales donde un insecto utilice más de un hospedero en simpatría (Agrawal 2000, Caswell 1983, 2001, Larsson *et al.* 2000, Awmack & Leather 2002)

Un interesante grupo de insectos fitófagos que exhibe uso de plantas hospederas desde la monofagia hasta la polifagia, es la familia Membracidae (Hemiptera), la cual se encuentra altamente diversificada (ca. 3200 especies) (Dietrich & Deitz 1991, Wood 1993a, Cryan *et al.* 2004, Lin 2006). A pesar de su distribución cosmopolita, la mayor riqueza de especies se concentra en los trópicos de Centro y Sudamérica (Lin 2004), mostrando patrones de especialización ecológica en especies que pertenecen principalmente a las familias Fagaceae, Asteraceae, Rubiaceae y Solanaceae (Wood & Olmstead 1984, Wood 1993b, Lopes 1995,

Sattman & Cocroft 2003). Se ha encontrado una mayor frecuencia de especies restringidas al uso de una o de pocas especies de plantas hospederas a alturas mayores que 2000 msnm (Richter 1954, Wood & Olmstead 1984, Sattman & Cocroft 2003).

1.1 Biología y Ecología del membrácido *Alchisme grossa* (Hemiptera: Hoplophorionini)

Alchisme grossa (Hoplophorionini) es una especie subsocial, es decir, las hembras realizan un complejo cuidado maternal activo de sus huevos y ninfas y, a diferencia de muchos otros membrácidos, esta tribu no presenta mutualismos con otras especies (e.g. hormigas). El grupo familiar (Madre + huevos o ninfas) se mantienen usualmente agregado bajo el cuidado de la madre incluso hasta que las ninfas se convierten en adultos. El cuidado maternal se materializa en tareas tales como: a) protección de los huevos, donde la madre recubre su ovipostura con una secreción glandular y se posa sobre ella durante todo su desarrollo, b) facilitación alimenticia para las ninfas, al perforar la madre el tallo o raquis de la hoja donde las ninfas se alimentan, c) defensa antidepredatoria, donde la madre ataca a cualquier potencial amenaza con patadas y vibraciones alares. Durante su desarrollo, las ninfas forman grupos alargados con la madre vigilante generalmente en uno de los extremos del grupo. En plantas que contienen varias familias, se ha observado en diversas ocasiones que las ninfas forman grupos mixtos entre familias a partir de la

culminación del tercer estadío, punto donde la autonomía de las ninfas les permite mayor independencia y movilidad; sin embargo, en la mayoría de los casos, las madres permanecen adyacentes a estos grupos en estado de vigilancia aparente. A lo largo de todo su desarrollo, las ninfas son incapaces de moverse a otra planta, motivo por el cual están obligadas a permanecer y alimentarse en la planta seleccionada por sus madres un promedio de 45 días. Cabe remarcar que los machos no ejecutan tarea alguna relacionada con el cuidado ninfal y son observados de forma esporádica alimentándose en las plantas. Las ninfas pasan por un total de 5 estadíos para luego convertirse en adultos; estos últimos permanecen aun en la planta un promedio de 8 días hasta que el pronoto que los cubre termina su esclerotización y las alas están completamente funcionales para el vuelo. Su patrón de movimiento se basa principalmente en vuelos cortos de pocos metros; sin embargo, se ha observado una alta precisión en su capacidad de vuelo (Torrico Bazoberry *et al.* 2014).

En Bolivia, la presencia de *A. grossa* ha sido reportada principalmente en la zona de los bosques nublados de la Yunga entre los 2000 y 2500 msnm. Este membrácido utiliza de forma exclusiva dos especies de planta hospedera de la familia Solanaceae (*Brugmansia suaveolens* y *Solanum ursinum*) en la localidad de Incachaca, a pesar de que esta zona está caracterizada como un "hot-spot" de diversificación de la familia Solanaceae (Olmstead 2013), contando con al menos 25 especies de esta familia en la zona, la mayoría de ellas del género *Solanum* y al

menos 3 del género *Brugmansia* (Torrico Bazoberry *et al.* 2014). En ambas plantas hospederas se ha observado una diversidad de depredadores atacando a ninfas y adultos, además, al menos una especie de ácaro que parasita adultos y ninfas de forma esporádica en ambas plantas hospederas (detalle en resultados del capítulo 2).

Experimentos de marcaje y recaptura han demostrado la existencia mayoritaria de un patrón de uso de hospederos alternativos en las poblaciones de *A. grossa* en estudio, ya que el 95 % de las recapturas y el 100 % de las hembras con oviposturas fueron realizadas en la misma especie hospedera en la cual el individuo se había desarrollado. Por otra parte, en estas poblaciones la duración del ciclo de desarrollo desde huevos hasta adultos es un 30 % más largo en *B. suaveolens* que en *S. ursinum.*

En relación con las especies hospederas de *A. grossa*, los compuestos secundarios propios de cada género de planta, principalmente aquellos frecuentemente relacionados con interacciones ecológicas y especialización en insectos (p.e., alcaloides), son producidos por vías metabólicas muy distintas (alcaloides derivados del tropano vs. glicoalcaloides esteroidales) y por lo mismo su interacción con los insectos puede variar en gran medida (Friedman 2006, Alves *et al.* 2012, Olmstead 2013). Esto constituye un actor de diferenciación entre los individuos que se desarrollan sobre una u otra planta hospedera.

Finalmente, las características microambientales ofrecidas por cada especie hospedera para el desarrollo de *A. grossa*, por ejemplo, temperatura y humedad relativa, es muy distinta entre ambos hospederos, y estarían relacionadas con la diferenciación en rasgos conductuales y atributos demográficos de *A. grossa* en cada hospedero, e.g. cohesión social, mortalidad, duración de estadíos (Torrico Bazoberry *et al.* 2014).

1.2 Problema

Considerando los antecedentes presentados, la asociación de *A. grossa* con sus dos especies hospederas corresponde principalmente a un escenario de uso de hospederos alternativos, es decir, los individuos de *A. grossa* desarrollados sobre uno u otro hospedero se reproducen y se dispersan mayoritariamente sobre la especie de planta hospedera en la que se desarrollaron, lo cual conduce a preguntarse si es exclusivo o no el patrón de uso de hospedero previamente observado, qué mecanismos están involucrados cuáles son las consecuencias asociadas a este patrón. En consecuencia, las preguntas abordadas en esta investigación son las siguientes:

El patrón de uso de hospedero observado en *A. grossa*:

1) ¿obedece a una selección directa de hospedero? Es decir, los individuos de *A. grossa* ¿se dispersan exclusivamente entre individuos de la especie hospedera de desarrollo, o existe también selección indirecta que involucra movimiento interhospedero?

2) ¿está relacionado con la potencial presencia de fidelidad alimenticia y/o con el secuestro de metabolitos secundarios por parte de los insectos?

3) ¿se mantiene en el tiempo gracias a que ambos ambientes constituyen escenarios igualmente favorables para la adecuación biológica de los individuos que los utilizan, a pesar de las diferencias fenotípicas que puedan presentar como sustratos?

4) ¿ha conducido a una estructuración genético-poblacional de los insectos que se desarrollan en cada hospedero alternativo?

1.3 Hipótesis

H1: El patrón de uso de hospederos alternativos está mediado por una selección directa de la especie hospedero en la cual se desarrollaron previamente los individuos (huevo-adulto), lo cual se verá reflejado en el patrón de dispersión intraespecífico de los individuos de *Alchisme grossa.*

H2: Los individuos desarrollados sobre una especie de planta hospedera exhiben fidelidad alimenticia a la misma, tomando en cuenta que individuos desarrollados

en cada hospedero incorporarán diferencialmente compuestos específicos de la química de su planta.

H3: La tasa de crecimiento poblacional de los individuos desarrollados en uno u otro hospedero no varía significativamente, dado que ambos hospederos representan ambientes igualmente favorables para el desarrollo de los individuos en ellos.

H4: Los individuos que se desarrollan sobre los distintos hospederos exhiben diferencias en cuanto al nivel de estructuración genético poblacional, la cual se encuentra asociada al patrón de uso de hospederos alternativos en *Alchisme grossa.*

1.4 Objetivo general

Estudiar el patrón de uso de especies hospederas de desarrollo por parte de *A. grossa* desde el punto de vista de sus procesos y consecuencias, a nivel ecológico y poblacional.

1.5 Objetivos específicos

1) Caracterizar el patrón de dispersión de individuos de *A. grossa* a niveles intra e interhospedero, utilizando adultos desarrollados sobre ambas plantas, y evaluar el nivel de fidelidad alimenticia por parte de *A. grossa* hacia sus hospederos de desarrollo.
2) Explorar la tasa de crecimiento poblacional asociada a los individuos de *A. grossa* desarrollados sobre uno u otro hospedero, con el fin de indagar la calidad del ambiente que constituye cada planta hospedera para la adecuación biológica de los individuos a nivel poblacional.
3) Caracterizar la relación entre la química de las plantas hospederas y la de los individuos de *A. grossa* desarrollados en cada una de ellas.
4) Determinar la ocurrencia de estructuración genético - poblacional en *A. grossa* asociado al uso de ambos hospederos, para evaluar la existencia de diferenciación genética y la magnitud de flujo genético entre individuos desarrollados en cada uno de los hospederos.

Estos objetivos son desarrollados en capítulos individuales, presentados a continuación, cada uno de los cuales incluye antecedentes teóricos específicos, la metodología utilizada, los resultados detallados y su respectiva discusión . Luego sigue una discusión general,y finalizando con las conclusiones de la investigación.

2. CAPÍTULO 1: DISPERSION PATTERNS AND FEEDING FIDELITY IN THE NEOTROPICAL MEMBRACID *Alchisme grossa* (Hemiptera) IN A CLOUD FOREST OF THE BOLIVIAN YUNGAS

2.1 Abstract

Phytophagous insects constitute a large proportion of entomological diversity in the world, where most of them are specialized at some level in a reduced number of hosts. The question about how ecological specialization can evolve when more than one host occur in sympatry is still under research. The evolution of philopatry seems to be the first step in this process, including patterns of dispersal and feeding preference towards the developmental host plants in phytophagous insects. Treehoppers constitute an ideal group to investigate host fidelity, since all the members of this group are strictly phytophagous and show patterns of host specialization in different groups of plants. *Alchisme grossa* (Hoplophorionini) is a subsocial treehopper specialized mostly on solanaceus plants, in the bolivian Yungas *A. grossa* utilizes *Brugmansia suaveolens* and *Solanum ursinum* - both Solanaceae - as host plants. We test using *A. grossa* the feeding preference and acceptance with behavioral bioassays, besides we characterize their natural patterns of dispersion in the field using mark recapture techniques. On both kinds of experiments, we controlled the developmental origin of insects. Results suggest feeding preference for developmental host plants, but on the other hand, individuals accept to feed on any of them in absence of chance to choose

(acceptance test). Dispersion patterns showed that *A. grossa* prefer to move and oviposite in the same host plant species of development, being females more mobile than males. Results showed the existence of philopatry mostly based on behavioral preference of individuals, this pattern can be related with imprinting processes during nymphal instars in *A. grossa* or with chemical interaction with respective hosts.

2.2 Introduction

Phytophagous insects constitute one of the most diverse and specialized groups on earth (Bernays & Chapman 1994, Caillaud *et al.* 2000, Schoonhoven *et al.* 2005), which depends on plants for different biological and ecological processes (e.g. reproduction, feeding, refuge) (Chittka & Thomson 2004, Schoonhoven *et al.* 2005, Speight *et al.* 2008). Insect – plant interactions generate a diversity of positive and negative effects for any or both groups, as well as no effects. Different attributes of plants and insects have evolved under the context of ecological interactions, where in many cases an original trait (such as, a secondary metabolite or a mechanical trait) involved in plant response against insect attack, later becomes a signal for plant recognition or acceptance by specialized insects (e.g. Swallowtail butterflies and Aristolochiaceae) (Bernays & Chapman 1994, Nishida 2002, Schoonhoven *et al.* 2005, Després *et al.* 2007).

Most phytophagous insects are specialized in a reduced taxonomic spectrum of plants, (Bernays & Chapman 1994, Bernays 2001, Schoonhoven 2005), host selection by insects being a vital process highly related with their survivorship and reproduction; in this scenario, insects need specific traits to recognize and discriminate potential host-plants, implying that sensory related traits must preexist in the insect (Futuyma & Moreno 1988; Jaenike 1990, Berlocher & Feder 2002, Powell *et al.* 2006). The colonization of a new host often requires behavioral changes in insects, and after this point, natural selection can act on characters expressed in the new environment (e.g. morphological, physiological); after the arrival of insects to a new substrate they are confronted with plant barriers and physiological and morphological traits of insects allow or limit their subsistence (Futuyma & Moreno, 1988). Then, colonization of a new host can exert a pressure which produces differentiation in traits related to host-plant use, influencing traits of insects living on them and promoting ecological specialization (Caillaud & Via 2000, Janz *et al.* 2001, Funk *et al.* 2002, Matsubayashi *et al.* 2009, Caillaud & Via 2012).

An important question is how ecological specialization can occur when both original and new hosts plants grow in sympatry, i.e. when reciprocal movements between hosts may occur (Wood *et al.* 1999). Two scenarios may explain cases where the same insect species is observed using more than one host in sympatry: a) the alternation of hosts, i.e. the same group of insects moves between different

plant species used as hosts, or b) the use of alternative host-plants, i.e. groups of an insect species use mainly one host-plant species and another group uses, albeit less frequently, a different plant species, from those present in their habitat. It may be speculated that the second scenario follows the first one, i.e. at the beginning of the specialization process, insects belong to a single group who colonizes a new plant species. Various ecological processes can occur during the evolution of ecological specialization as product of the use of alternative host-plants in sympatry, for example, a) the evolution of fidelity towards the developmental host-plant, b) the development of coordination between insect and plant phenology, and c) assortative mating on each host-plant, among others (Futuyma y Moreno 1988, Jaenike 1990; Schoonhoven 2005). These processes can finally cause a reduction in gene flow between groups of insects living on each host and promote the occurrence of sympatric or ecological speciation (Futuyma & Moreno 1988, Caillaud *et al.* 2000, Berlocher & Feder 2002, Drès & Mallet 2002, Caillaud & Via 2000; Funk *et al.* 2002, Matsubayashi *et al.* 2009).

One of the first steps occurring during the ecological specialization process is, host-plant fidelity (preference of adult insects to live and reproduce in the developmental host-plant), which involves the evolution of behavioral discrimination based on sensorial recognition during host selection (Bernays & Chapman 1994, Powell *et al.* 2006, Matsubayashi *et al.* 2009). This is a consistent pattern observed in different insect groups (e.g. Hemiptera, Coleoptera, Diptera,

among others) (Tilmon *et al.* 1998, Jaenike 1990, Singer *et al.* 1992, Barron 2001, Xue *et al.* 2009, Caillaud & Vía 2012), and involves genetic and epigenetic regulation (Futuyma & Peterson 1985, Prokopy *et al.* 1982, Boller *et al.* 1998, Agrawal 2000, Barron 2001). Different theoretical models have explained the evolution of preference in terms of feeding and reproduction value for insects. Models of preference – performance or host ranking models propose that preference for a host is positively related with a higher performance by the insect or, on the other hand, a higher hierarchy in and their preference to those hosts. Nevertheless, conclusive patterns about the certainty of these models are still not empirically proven in nature (Jaenike 1988, Courtney *et al.* 1989, Agrawal 2000, Scheirs 2002).

Treehoppers (Hemiptera, Membracidae) are an ideal group to investigate the evolution of host fidelity, since they perform on their host-plant most of their biological processes (e.g. feeding, mating, oviposition, development). They show different levels of diet breadth including host specialization in some families of plants (e.g. Solanaceae, Fagaceae and Fabaceae) (Wood & Olmstead 1984, Wood 1993b, Sattman & Cocroft 2003). *Alchisme grossa* (Hoplophorionini) is a sub-social treehopper (maternal care + nymphal aggregation) (Lin *et al.* 2004, Camacho *et al.* 2013, Torrico-Bazoberry *et al.* 2014) specialized mostly on solanaceus plants, a fact that has been suggested to be linked directly with plant chemistry (Wood 1993, McKamey & Deitz 1996, Pinto unpublished data). *A. grossa* utilizes *Brugmansia*

suaveolens (Humb. & Bonpl. ex Wild) Bercht. & C. Presl (Solanaceae) and *Solanum ursinum* (Rusby) (Solanaceae) as host-plants; these species occur in sympatry in the cloud forests of the Bolivian yungas (Torrico-Bazoberry *et al.* 2014), and females of *A. grossa* oviposit and take care of nymphs along their development on both species. Individuals of *A. grossa* are limited to remain and develop through all nymphal stages and part of adult stage (c.a. 80 days) in the host-plant individual where they were borne. After that, adults have the capacity to move to a new host individual, a factor that can involve a strong imprinting effect from the host-plant on insects. *A. grossa* females have the chance to use either a *B. suaveolens* or *S. ursinum* individuals to oviposit since both hosts grow interspersed in the cloud forests of Bolivian yungas, a zone characterized by a high Solanaceus diversity (Torrico-Bazoberry *et al.* 2014). Field experiments suggest that individuals developed on each host continue using the same species of host plant along time (personal observations).

These factors led us to wonder if host fidelity has already emerged in the interaction between *A. grossa* and their hosts, and if so which traits from insects are involved in the process? Controlling the origin of development of *A. grossa* (*B. suaveolens* or *S. ursinum*), we propose two different approximations to investigate those questions: a) to characterize the level of feeding fidelity in terms of preference (which host is preferred depending on the substrate of development?) and acceptance (which is the capacity of the insect to accept each of the host-plants

depending on the substrate of development?) and b) to characterize natural dispersion patterns of *A. grossa* in the field, in order to know if they remain continuously on the same host-plant of development or if they move periodically between host-plants. Both aspects can shape the level of host fidelity developed by *A. grossa.*

2.3 Methodology

Study area and data collection

Fieldwork was performed at Incachaca (near Cochabamba, Bolivia 17º15'17"S - 65º48'54"W; 2359 m.a.s.l.), an area belonging to the Yungas Biogeographical Province (Navarro & Maldonado 2002). The study area was composed by two high mountain cloud forests patches (Locations A and B) characterized by a heavy rain season (3700 mm per year concentrated from October to April) and a wide altitudinal variation (600 – 3000 masl) (Navarro & Maldonado 2002). Both patches are distanced by 3 km at the same altitude, each of them including populations of *A. grossa* (AG) and both of its hosts, *S. ursinum* (SU) and *B. suaveolens* (BS). For feeding fidelity bioassays, *A. grossa* individuals were reared on BS and SU at A location. In the case of dispersion patterns protocols, individuals used for fieldwork came from both host-plants from both locations. Data collection was carried out from December 2013 to March 2014.

Feeding fidelity

In order to assess the existence and level of host-plant feeding fidelity by *A. grossa* to *B. suaveolens* or *S. ursinum* in relation with the developmental substrate, we characterized patterns of feeding preference and acceptance of both host-plants while controlling the substrate of development of insects. Host-plant preference involves a choice situation where the insect discriminates, according to its individual preference, and chooses an item among those available in the environment (Courtney *et al.* 1989, Singer, 2002). On the other hand, acceptance involves a sort of no-choice situation (Since, the insect can still decide to feed or not feed), where a single item is available; thus, the individual has no possibility to choose an item and, depending on its motivation (i.e: the necessity to oviposit, feed, or mate), may use the presented item to different extents (Singer 2002, Schoonoven *et al.* 2005). The evaluation of host-plant acceptance allowed us to determine if an insect is able to use a specific item even if it prefers another species that is not available in the environment. In this context, host-plant preference was evaluated presenting two plants (one BS and one SU) to a single AG adult (male or female) developed on BS or SU; thus, four treatments (N=25 each) were performed: 1) BF: AG female developed on BS, 2) BM: AG male developed on BS, 3) SF: AG female developed on SU, and 4) SM: AG male developed on SU. For plant acceptance, a single plant of BS or SU was presented to a single AG adult (male or female) developed on BS or SU; thus, eight treatments (N= 20 each) were performed: 1) BFB: Female AG developed on BS presented to a BS plant, 2) BFS: Female AG

developed on BS with SU plant presented, 3) BMB: Male AG developed on BS with BS plant presented, 4) BMS: Male AG developed on BS with SU plant presented, 5) SFS: Female AG developed on SU with BS plant presented, 6) SFB: Female AG developed on SU with SU plant presented, 7) SMS: Male AG developed on SU with BS plant presented and 8) SMB: Male AG developed on SU with SU plant presented. Behavioral characterization for both kinds of bioassays (preference and acceptance) was based on preliminary field observations and previous reports for this group (e.g.: Powell *et al.* 2006, Caillaud & Via 2000, Singer et al. 1992), and included the following behaviors: i) latency, when the stylet of insect was not inserted into plant tissue and the individual stayed immobile or walked, ii) probing, when a half of the stylet was inserted into the plant tissue, and iii) feeding, when the stylet was totally inserted into the vegetable tissue. For host preference bioassays, the behavior and position (over any of both plants) of an individual were recorded using an instantaneous sampling every 15 minutes for 5 hours (Lehner, 1998). In the case of plant acceptance bioassays, behavior and position (over or out of the plant) were recorded using a the continuous sampling method (Lehner, 1998) for 2.5 hours using the JWatcher 1.0 software (Blumstein & Daniel 2007). Bioassays were performed in transparent acrylic boxes (preference: 75 x50 x 50 cm; acceptance: 30 x 50x 30 cm) surrounded with Styrofoam. Temperature and humidity were registered with a termo-humidimeter (Extech 4465CF) at the beginning and end of each bioassay.

Dispersion patterns of *A. grossa*

Natural dispersal patterns of *A. grossa* were assessed using a mark-recapture technique (Hagler & Jackson 2001); after emergence, *A. grossa* individuals remain on the host-plant where they were borne for at least 8 days (Torrico-Bazoberry *et al.* 2014); this time period was used to capture and mark individuals. This procedure was carried out in two patches of cloud forest (locations A and B), which contain SU and BS individuals in simpatry. In these patches, 7.5 x 7.5 meters grids were established: 56 grids in location A (3150 m^2) and 64 grids in location B (3600 m^2). All host-plant individuals on each location were marked and mapped. Each plot was scanned every 10 days (11 scans in total) for four months. During fieldwork clutches and nymphs founded were monitored over time to identify the developmental host of newly emerged adults. All membracids were set free after registering sex, host species, and position in the grid.

Statistical analyses

Feeding fidelity

Host-plant preference was estimated comparing the number of events of each behavior performed on each host-plant during bioassays. Using a hierarchical Bayesian analysis for count data, that includes 10000 Monte Carlo Markov Chains (MCMC) (BayesPref package, Gompert & Fordyce 2013), preference for host-plant A was expressed as the probability that an alternative host-plant were preferred more or equal than host-plant A (Fordyce et al. 2011) i.e.: for a p-value = 0.05, the

individual had a 0.05 probability to prefer host-plant B rather than host-plant A; thus, the lower the p-value of A, the higher the preference for this item.

Four different grouping schemes were examined to determine which populations might share the same pattern of preference: a) without grouping (BF, BM, SF and SM separately), b) grouping treatments by sex (BF + SF and BM + SM), c) grouping by developmental host-plant (BF + BM and SF + SM), and d) grouping all treatments as a host-plant preference of the species (BF + BM + SF + SM). For each model, the strength of preference for each host-plant species was determined by distributions of preference parameters after 10000 iterations of MCMC followed by a burn-in of 1,000 generations (for more details about these parameters, see Fordyce et al. 2011).

Plant acceptance was analyzed comparing the proportion of individuals that used the presented host-plant vs. those that did not, between treatments. Comparisons between treatments were carried out for each behavior separately, using a Marascuilo's Multiple Comparisons Analyses (http://www.statstodo.com/MultiProp_Pgm.php). To detect any variation in behaviors due to the host-plant presented, the total duration of each behavior by individuals that had used the presented plant was compared between treatments with an ANOVA on ranks using SigmaPlot 11.0 software.

Dispersion Analyses

Sex ratios of *A. grossa* were determined for each location and host. Proportions of recaptures per sex were compared between locations and sexes using a Chi-square test. Mobility of *A. grossa* individuals was characterized as the ratio between individuals that stayed in the plant of development and individuals that moved to another plant; comparisons between these ratios between sexes and host-plants of development were performed using Chi-square, and Marascuilo's Multiple Comparisons Analyses (Westfall & Wolfinger 1997).

To assess dispersal capability of *A. grossa* individuals, we calculated the Maximum Distance Traversed (MDT) and Mean Activity Radius (MAR) (Samietz & Berger 1997). As home range estimator, we calculated the Minimum Convex Polygon (MCP) (Powell 2000). MDT was calculated as the maximum distance reported of all recaptured individuals on each location. MAR was calculated as the arithmetic mean of all straight-line distances between the first observation and the recaptures of the individuals that hatched per plant (123 plants for *B. suaveolens* and 117 for *S. ursinum*). MCP was calculated as the area within the most peripheral recaptures (at least five points) of an individual (15 individuals of *B. suaveolens* and 7 of *S. ursinum*). Comparisons of these variables were carried out taking into account host of development and sexes using Kruskal-Wallis and Mann-Whitney tests.

Direct or indirect host-plant selection was assessed as the likelihood of individuals to be found on the alternative developmental host-plant species at least once over

time. This was calculated as the ratio of the individuals recaptured on the developmental host-plant and the proportion of individuals recaptured on the alternative host-plant at least once. Comparisons of these data were carried out using all recaptures per host with Binomial analyses to identify differences in direct vs indirect plant selection.

2.4 Results

Feeding fidelity

For all behaviors, the order of model adjustment (according to ascendant Deviance Information Criterion (DIC)) was: "grouping by sex" (lowest DIC), followed by "grouping all treatments", "without grouping", and finally, "grouping by developmental host-plant" (highest DIC) (Table 1).

Table 1. Host-plant preference of *A. grossa* for each behavior (inactivity, probing and feeding. DIC values are presented for each behavior on each model. Preference (Pref.) for both host-plants is associated to the probability (Prob.) that the alternative host-plant is preferred more or equal that the host-plant evaluated.

1a. Host-plant preference of *A. grossa* grouping by sexes

1a. Grouping treatments by sexes							
		Females (BF+SF)			Males (BM + SM)		
Behavior	**DIC**	**N**	**Preference**	**Probability**	**N**	**Preference**	**Probability**
Feeding	-218,171	39	B>S	0.02<0.98	38	B>S	0.00<1
Probing	-18,04338	28	B>S	0.45<0.55	33	B>S	0.30<0.70
Inactivity	-12,69834	28	B>S	0.19<0.81	32	S>B	0.35<0.65

1b. Host-plant preference of *A. grossa* grouping all treatments

1b. Grouping all treatments (BF + BM + SF + BM)				
Behavior	**DIC**	**N**	**Preference**	**Probability**
Feeding	-26,8464	77	B>S	0.02<0.98
Probing	-31,5949	61	B>S	0.30<0.70
Inactivity	-9,44848	60	B>S	0.36<0.64

1c. Host-plant preference of *A. grossa* without grouping treatments

1c. Without grouping treatments												
Behavior	DIC	BF (n=19)			BM (n=19)			SF (n=22)			SM (n=20)	
		N	Pref.	Prob.	N	Pref.	Prob.	N	Pref.	Prob.	N	Pref.	Prob.
Feeding	-663,6	19	B>S	0.01<0.99	19	B>S	0.00<1	22	S>B	0.35<0.65	20	S>B	0.08<0.92
Probing	-72,5	14	B>S	0.02<0.98	15	B>S	0.06<0.94	14	S>B	0.02<0.98	18	S>B	0.26<0.74
Inactivity	-127,6	14	B>S	0.02<0.98	17	B>S	0.01<0.99	14	S>B	0.23<0.77	15	S>B	0.01<0.99

1d. Host-plant preference of *A. grossa* grouping treatments by developmental host plant

1d. Grouping treatments by developmental host-plants							
		Individual from *B. suaveolens*			Individual from *S. ursinum*		
Behavior	DIC	(BF + BM)			(SF + SM)		
		N	Preference	Probability	N	Preference	Probability
Feeding	-89,2661	41	B>S	0.00<1	36	S>B	0.10<0.90
Probing	-4,1244	29	B>S	0.01<0.99	32	S>B	0.04<0.96
Inactivity	160,0452	31	B>S	0.01<0.99	29	S>B	0.01<0.99

In the "Grouping by sex" treatment (regardless of the developmental host-plant), females (BH + SH, $P = 0.02$) and males (BM + SM, $P < 0.001$) showed a strong and higher preference for feeding on BS than feeding on SU (Table 1a). In the case of "Grouping all treatments", the pattern was similar suggesting a stronger and higher preference for feeding on *B. suaveolens* than feeding on *S. ursinum* ($P = 0.02$, Table 1b). Analyzing each treatment separately, there was a clear preference in all treatments for their developmental host-plant for all behaviors (Table 1c). Finally, in the case of "grouping by developmental host-plant" (regardless of their sex),

individuals of each host-plant had a stronger preference for their own developmental host-plant in all behaviors (Table 1d). Although the last two schemes (without grouping and grouping by developmental host-plant, see Table 1c and 1d) suggest feeding fidelity for the host-plant of development, conclusions have to be carefully formulated since both schemes had the highest DICs. Besides, since "Feeding" is the most representative behavior of host-plant preference evaluated in this study, density of probability as a function of population preference was represented for the four models (Fig. 1).

In a non-choice situation (acceptance), individuals of all treatments were able to use both host-plants since no significant differences in individual proportions were found between treatments for any behavior (d.f. = 7, P = > 0.05 n.s.; Feeding: X^2 = 9.9244, Probing: X^2 = 5.6145, Latency: X^2 = 5.7482). In the same way, no differences among treatments were found in the total duration of each behavior suggesting that different behaviors were not influenced by the species of host-plant presented (d.f. = 7, P = > 0.05 n.s.; Feeding: H = 1.81,Probing: H = 5.04, Latency: H = 3.74) (Table 2).

Dispersion

Altogether, 1905 *A. grossa* individuals were captured and marked. Recaptures reached 28.8% and 18.49% for locations A and B, and reached 27.01% for *B. suaveolens* and 18.44% for *S. ursinum,* respectively.

Overall sex ratio of *A. grossa* individuals captured was 0.83:1 males:females. Likewise, sex ratios within locations and host species were biased to females: ranging from 0.78 to 0.85 males per female (Table 3). Chi-square tests showed statistical differences between sexes for locations and hosts ($P<0.001$); females were always more abundant than males.

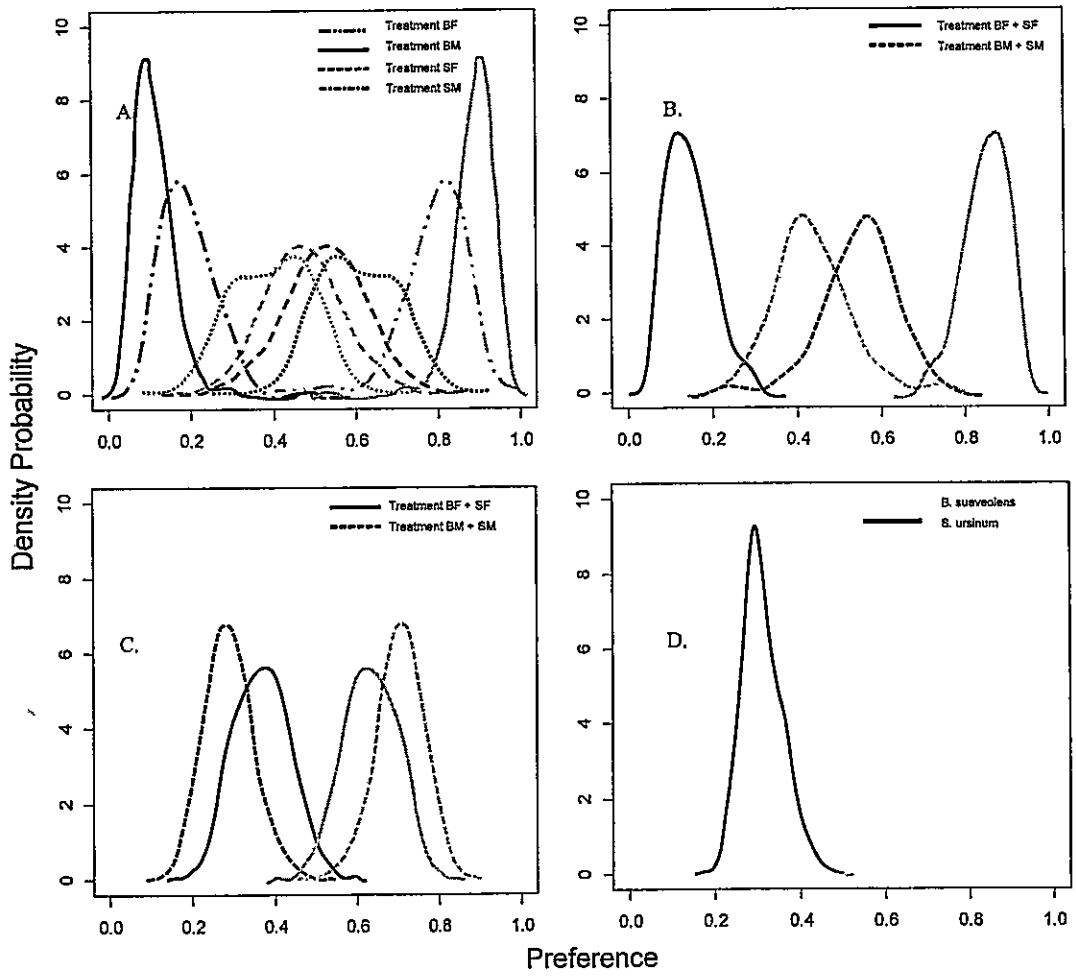


Figure 1. Host-plant preferences under different population scenarios. Curves indicate posterior density for population-level preference for two host-plant species (grey curves: *B. suaveolens*, Black curves: *B. suaveolens*) for four models: a) without grouping, b) grouping by sexes, c) grouping by host-plant of development, and d) grouping all treatments. Joining treatments are specified in legend. Posterior densities estimated from 10000 MCMC steps following a burning of 1000 generations.

Table 2. Number of individuals that performed and did not perform each behavior, and duration (mean ± standard error, minutes) of each behavior in Plant Acceptance bioassays. Marascuilo and One-Way ANOVA on ranks statistics are presented including values of Chi-Square = X^2, Kruskal-Wallis = H, degrees of freedom (d.f.), and *P*-value (P).

4a. Feeding					
Treatment	**Performed**	**Did not perform**	**Marascuilo Statistics**	**Duration**	**One-Way ANOVA Statistics**
BHB	9	13	$X^2 = 9.9244$, d.f. = 7, $\alpha = 0.1929$	33.2 ± 5.4	H = 1.81, d.f. = 7, P = 0.970
BHS	8	12		39.3 ± 7.3	
BMB	11	9		37.4 ± 8.1	
BMS	11	9		36.1 ± 8.4	
SHB	14	7		33.7 ± 5.9	
SHS	17	5		34.5 ± 9.5	
SMB	9	11		43.3 ± 9.7	
SMS	11	9		40.3 ± 9.5	

4b. Probing					
Treatment	**Performed**	**Did not perform**	**Marascuilo**	**Duration**	**Statistics**
BHB	14	8	$X^2 = 5.6145$, d.f. = 7, $\alpha = 0.5854$	50.1 ± 12.4	H = 5.04, d.f. = 7, P = 0.655
BHS	15	5		43.0 ± 9.1	
BMB	15	5		32.4 ± 8.9	
BMS	15	5		27.1 ± 7.1	
SHB	18	3		40.9 ± 8.3	
SHS	19	3		42.8 ± 9.7	
SMB	17	3		52.6 ± 15.4	
SMS	14	6		48.1 ± 9.2	

4c. Latency					
Treatment	**Performed**	**Did not perform**	**Marascuilo**	**Duration**	**Statistics**
BHB	21	1	$X^2 = 5.7482$, df = 7, $\alpha = 0.5694$	72.6 ± 16.7	H = 3.74, d.f. = 7, P = 0.81
BHS	20	0		61.3 ± 14.9	
BMB	20	0		66.0 ± 12.5	
BMS	20	0		53.4 ± 16.1	
SHB	20	1		50.9 ± 12.4	
SHS	22	0		76.4 ± 9.9	
SMB	20	0		58.5 ± 9.2	
SMS	20	0		58.7 ± 11.7	

Table 3. Sex ratio of *A. grossa* individuals marked per host-plants and locations. Chi square (χ^2) analyses showed statistical differences stating females to be more abundant than males within host species and locations (χ^2= Chi square value; df= degrees of freedom; P= p-value).

	Sex Ratio (♀/♂)	**χ^2, df, *P***
B. suaveolens	0.78:1	9.12, 1, <0.01
S. ursinum	0.85:1	8.08, 1, <0.01
Location A	0.80:1	6.06, 1, <0.01
Location B	0.84:1	10.60, 1, <0.01

Mobility pattern analyses indicate that *A. grossa* individuals are sedentary, and tend to stay in the plant of development instead of moving to another plant (independent of host-plant species). Comparisons between individuals that stayed vs. those that moved to other plants showed a greater percentage (58.5%) of individuals stayed in their developmental plant individuals (z= 4.36, *P*<0.01). A

difference between sexes, where females are more mobile than males was also registered (z= 2.69, $P<0.01$). When we included host-plant species as factor in the analyses, results showed that *A. grossa* individuals developed on *S. ursinum* are predominantly sedentary (68.25%) ($\chi^2=17.34$, $P> 0.05$ df=1); on the other hand, in the case of *B. suaveolens,* no statistical differences were found ($\chi^2=0.01$, $P=0.9$, df=1).

Similarly, an overall comparison of individuals' mobility showed statistical differences (z=2.38, $P=0.02$) that point to *A. grossa* individuals developed on *B. suaveolens* beibg more mobile than those developed on *S. ursinum.* Likewise, Marascuilo's post hoc analysis supports this result, and states differences of males vs. males, females vs. females between hosts, but no differences between sexes within hosts (overall $\chi2= 42.74$, df= 3, $P<0.01$) (Table 4).

Dispersion parameters and home range estimators were similar between host-plant species. Maximal distance reported (MDR) was 80 meters for females and 60 meters for males developed on *S. ursinum*, and 82.5 meters for females and 52.5 meters for males developed on B. *suaveolens.* Mean activity radius (MAR) was 15.50 ± 1.50 meters for individuals developed on *S. ursinum*, and 18.48 ± 1.24 meters for individuals developed on *B. suaveolens.* Although Mann-Whitney tests showed no differences of MAR between sexes in total, nor sexes within hosts ($P>0.05$), the general comparison between hosts showed a statistical difference (U=6154.50; $P =$

0.04), which indicates that *A. grossa* individuals from *B. suaveolens* exhibit a greater MAR than individuals from *S. ursinum*.

In the case of home range estimates, the Minimum Convex Polygon (MCP) showed a maximum traversed area of 1020.44 m^2, a minimum of 5.79 m^2, and an overall mean of 224.62 ± 55.07 m^2. Additionally, Mann-Whitney tests showed no differences of *A. grossa*'s MCP between locations or host-plant species. These results indicate that, in general, there are minimal differences of *A. grossa*'s dispersion parameters between sexes or host-plant of development, and that their dispersal capacity allows individuals to change host-plant species within the areas studied, although this was not observed under natural conditions.

Table 4. Mobility, dispersion parameters, and host fidelity of *A. grossa* individuals marked in *B. suaveolens* and *S. ursinum*. Comparisons of data between hosts and sexes for Mobility (% of dispersing individuals), Mean Activity Radius (MAR), and Minimum Convex Polygon (MCP) were carried out by Z-test (z), Marascuilo's post hoc analyses (χ^2), Mann-Whitney test (U), and Kruskal-Wallis test (H). For maximal distance traversed (MDT), and Host Fidelity (% of individuals that stayed in host of development) general values are presented.

	B. suaveolens		*S. ursinum*		**Between hosts**	**Between sexes**
	♂	♀	♂	♀		
Mobility (%)	49.6		31.85		z= 2.38 p=0.02	-
	47.76	65.38	23.53	33.57		X2=42.74, Df=3, α<0.001
MDT (m)	52.5	82.7	60	82.7	-	-
MAR (m)	18.48 ± 1.24		17.50 ± 1.50		U=6154.50 p=0.04	-
	19.22±2.26	18.52±1.90	16.92±2.28	20.52±2.67	-	H=3.27 p=0.41
MCP (m2)	177.88±68.29		324.75±87.10		U=31.00 p=0.14	-
Host Fidelity(%)	96.43		99.21		-	-

Host fidelity binomial analyses showed an overall fidelity to host of development in all comparisons ($P<0.01$). Of all recaptured individuals, 96.9% recaptures occurred in the same host-plant species, indicating that individuals preferred the host-plant species where they were born instead of the alternative host-plant. Likewise, in locality A and locality B individuals recaptured showed 98.7% and 95.2% host fidelity, respectively ($P<0.01$). When including host-plant species in the analysis, the results showed the same pattern; thus 96.4 % and 99.2 % of individuals marked in *B. suaveolens* and *S. ursinum,* respectively, were recaptured on the same host-plant species ($P<0.01$) (table 5). Additionally, host fidelity was not only observed through recaptures, but also through marked females that later on established new clutches on their host-plant of development. Thus, from 45 clutches found during fieldwork, 93.3% were laid in the host-plant specie of development; 33 (73.3%) of those new clutches were laid on another plant individual, and only 9 (20.0%) were laid on the same plant where the female was born, showing a clear preference for the host-plant species of development.

2.5 Discussion

Feeding fidelity in *A. grossa* showed two main patterns depending on the grouping treatment for data analysis. In the case of grouping by sex treatment, BS probed to be the preferred host-plant independently of the developmental host-plant or sex of insects: when all treatments were coalesced into a single group, preference was also directed to BS. In the other two grouping treatments –the weakest ones as judged by their higher DIC values- (without grouping and grouping by developmental host-plant), there was a tendency to prefer the developmental host-plant. On the other hand, bioassays on plant acceptance showed that *A. grossa* accepted any of the two host-plants if there is no other feeding alternative. In the case of dispersal analysis of *A. grossa,* sex ratios showed to be biased to females on both host-plants, where most individuals preferred to stay in the same plant where they were born in the case of SU and being more mobile in the case of individuals developed on BS. Following this pattern, MAR of males and females were similar within each host, but individuals developed on BS showed a greater MAR than those from SU. Home range of *A. grossa* individuals was similar between host-plants and sexes. These results indicate that, in general, there are minimal differences of *A. grossa*'s dispersion parameters between sexes or host-plants of development, and that its dispersal capacity allows them to change host-plant species for *A. grossa* individuals within the areas studied; nevertheless, individuals displayed fidelity to the developmental host-plant under natural conditions.

For treatments "without grouping" and "grouping by developmental host-plant", individuals preferred its developmental host-plant. However, such preference was maintained only by BM and BF treatments when they were combined with SH and SM treatments ("grouping all treatments" and "grouping by sexes") which changed its preference to the alternative host-plant, BS. Since the analysis performed with BayesPref use the preference level of individuals to calculate the population level preference (Fordyce *et al.* 2011), the change of preference pattern of SH and SM treatments could be related with the high preference of individuals developed on BS which dilutes the weak preference effect of individuals developed on SU. In this case, a logical question arises about why individuals from BS have a stronger preference for its developmental host-plant than those from SU.

Ecological context constitutes an important factor that constrains the evolution of host-plant use and preference patterns (Courtney *et al.* 1989, Futuyma & Moreno 1998, Schoonhoven *et al.* 2005). Different evolutive factors (e.g. historical patterns of host-plant use and specialization) could promote a sort of hierarchy between host-plants, biasing insect preference to those host-plants that showed the most ancient relationship with them. For example, an insect that almost never had contact with a specific plant -the "new host" - is able to use and even prefer it over its developmental host-plant species if the relationship between the insect species and the "new host" species is older but has been interrupted for a long time (Courtney *et al.* 1989, Singer 2002). Dating of *A. grossa* – host-plant relationship

remains unknown along Bolivian cloud forests; nevertheless, *Solanum* has radiated earlier and produced more species as *A. grossa*'s hosts than *Brugmansia* (McKamey & Deitz 1996, Camacho *et al.* 2013, Martins & Barkman 2005). This is supported by the fact that after a national search, five *A. grossa* populations have been observed using SU while only a single population was observed using also BS (population used in this study) (Pinto, personal communication), a pattern that suggests an older relationship with SU than with BS and consequently a potential preference for this host-plant species; nevertheless, BS seems to be the preferred feeding host-plant of *A. grossa*.

Although *Brugmansia* and *Solanum* belong to the same family, they are quite different in many traits (e.g. morphological, phenological, chemical attributes). For example, trichome density and tissue hardness is higher on SU than in BS, two factors that could affect substrate quality or difficulty for proboscis insertion by AG individuals (Wei *et al.* 2000, Pinto unpublished data). On the other hand, host-plant associated secondary metabolites could be involved in different behavioral insect responses since common solanaceus compounds, such as alkaloids, have been reported to be quite different between both genera (BS: tropane alkaloids; SU: steroidal alkaloids) acting in different ecological contexts associated with insect - plant interactions (Bernays & Chapman 1994, Schoonhoven *et al.* 2005). *Brugmansia* plants containing alkaloids derived from tropane that can act as phagostimulants, deterrents or in a defensive context for insects (e.g.

sequestration), while *Solanum* plants producing steroidal alkaloids seem to sustain fewer interactions (Wink 2003). Besides, the fact that *A. grossa* can sequester secondary metabolites from BS and not from SU, suggests a strong feeding preference for BS developed thorugh a durabe interaction and a recently colonized host-plant in the case of SU.

A strong relationship between genetic variation of membracids and the use of their host-plants has been reported as promoting diversification for the *Enchenopa* (Hemiptera) complex. In this process, a previous evolution of fidelity to developmental host-plants is also involved (Tilmon *et al.* 1998, Caillaud & Via 2012, Matsubayashi *et al.* 2011, Powell *et al.* 2006). On the other hand, learning capacities in some phytophagous insects are involved in diversification under a sympatric mode of speciation or, at least, in inter-population variation of some traits related to choice of host-plants. In the case of *A. grossa,* host-plant cues are easily learned on early stages of insect life, and since *A. grossa* offspring have low mobility, plant-related cues can have more chance to be acquired during egg and/or nymphal stages, conditioning in some way adult behavior and preference (Barron 2001, Papaj & Lewis 2012). All of these factors can be working together on *A. grossa* populations living and preferring each of their two alternative host-plants, but potentially in an earlier stage of development compared with *Enchenopa*, where possibly only behavioral barriers have been developed and are in charge of promoting host-plant fidelity.

Although few studies have been carried out in order to address dispersion parameters of membracids, Wood & Dowell (1985) reported for *Umbonia crassicornis* (Hoplophorionini) - and using almost the same mark-recapture technique- a mean of 24% of recapture success. Data reported in our study suggest a similar success in recapture with values between 18 and 29%. Sex ratio values of *A. grossa* populations were slightly female-biased in all cases (within locations and host-plants of development), similar to patterns observed in other species of membracids studied (Wood & Dowell 1985). Nevertheless, higher female-skewed values were also reported in the past (Masters *et al.* 1994). Trivers & Willard (1973) suggest that biased sex ratio could be influenced by the nutritional status of parent females at the time of reproduction, rearing more males only when nutritional status is good. Masters *et al.* (1994), however, indicates that female biased populations of *Umbonia ataliba* (Hoplophorionini) does not appear to be explained by this hypothesis, and that treehoppers are not known to have proximate control over progeny sex ratio. Moreover, quality of host-plant nutrients received by offspring may control sex ratios rather than parental factors, for instance, causing starvation and higher mortality of a certain sex in membracids, or alternatively, inbreeding and local-mate competition could favor an investment biased to females (Wood & Dowell 1984, Masters *et al.* 1994). In the case of *A. grossa*, differences found on host-plant quality could possibly explain skewed sex ratios since differences in performance and resource quality have been previously

reported for *A. grossa* developed over the same host-plants (Bazoberry *et al.* 2014, Pinto unpublished data).

A. grossa populations appear to have low vagility (59%), similar to other membracid populations which show clumped distributions (Wood & Dowell 1985, Funderburk & Mack 1989). The differences found between sexes (females seem to be more mobile than males) diverge from the findings of Wood and Dowell (1985) for *U. crassicornis* populations, where males are more mobile than females. At the same time, low vagility seems to provide an ideal scenario where close relatives can potentially mate between them; thus, differences in vagility between sexes could promote outbreeding (Wood & Dowell 1985). According to our results, female vagility could be influencing a potential outbreeding process on both host-plants, in addition to differences imposed by the host-plant species, since *A. grossa* individuals developed on *B. suaveolens* are more mobile than those developed on *S. ursinum*.

Values found for MDR, MAR and MCP are higher in comparison to the values recorded for other membracid species (Wood & Dowell 1985, Funderburk & Mack 1989), stating that *A. g*rossa has a relatively higher capacity of dispersion. However, our data showed no differences between sexes, in contrast to the differences in mean activity radius between sexes of *U. crassicornis* (Hoplophorionini) reported by Wood & Dowell (1985). In general, vagility and dispersion parameters give *A.*

grossa individuals the chance to change host-plant species over time, and also to mate with non-relatives developed over any host-plant species, but fidelity values reported (>93%) indicate that individuals prefer the host-plant species where they were born, in addition to the high preference of ovipositing females (93%).

We conclude that a clear pattern of host fidelity was evidenced for males and females of *A. grossa* using two solanaceus sympatric hosts which seems to be promoted mostly by behavioral traits since no physiological barrier was identified through acceptance bioassays; at the same time, mark and recapture protocols showed that insects remain on the developmental host-plant even though they the chance to move, mate or oviposit on the alternative sympatric host-plant. Moreover, BS seems to be more preferred by individuals developed on them than in the case of SU. This scenario makes one wonder about how important fidelity is as a premating barrier between individuals developed on the alternative host, a phenomenon involved in diversification of other phytophagous insects, and if that host fidelity has resulted in promoting the mating isolation of two populations, or in an extreme case, of two cryptic species living in sympatry.

3. CAPÍTULO 2: EFFECT ON VITAL RATES OF ALTERNATIVE HOST PLANT USE IN THE TROPICAL TREEHOPPER *Alchisme grossa* (HEMIPTERA)

3.1 Abstract

The ecology and evolution of host use patterns in phytophagous insects that use alternative host in simpatry could be best understood, if patterns of performance at individual and population level were studied together. Performance can be characterized from a population point of view using demographic parameters as proxies of group performance. *Alchisme grossa* is a subsocial treehopper that utilizes two alternative host plants in sympatry, *Brugmansia suaveolens* and *Solanum ursinum* (both Solanaceae), in the cloud forests of the bolivian yungas, and showing fidelity to the developmental host species. We modeled *A. grossa* finite rate of increase (λ) and performance on each host following females and their nymphs during all their development. At the same time we compare different characteristics of plants potentially involved in the ecology with the insect on each host. Finally, we characterize maternal care and their relation with nymphal survivorship. λ values and performance showed similar values between hosts, instead of many differences founded between characteristics of each plant species. At the same time, a strong relation between maternal care and nymphal survivorship was founded on both hosts, without differences in the duration of this trait between *B. suaveolens* and *S. ursinum*. Results are discussed in relation with

factors that could explain or balance costs and benefits of individuals living on each host species.

3.2 Introduction

In phytophagous insects, host plants are used in different contexts of their biology and ecology, where patterns observed in nature about the use of plants as nutritional resources and habitats are shaped by historical, physiological, morfological and ecological factors between others (Futuyma & Moreno 1988, Bernays & Chapman 1994, Jaenike 1990, Schoonhoven 2005). The effect of these factors is reflected in the level of restriction (or amplitude) of hosts used. In this sense, ecological specialization is widespread among species, being a rule more than an exception along examples of host use patterns described for phytophagous insects (Futuyma & Moreno 1988, Schoonhoven 2005). An important set of research has been performed looking for ecological and genetic mechanisms that produce specialized resource or habitat use (e.g., Berenbaum 1996, Caillaud & Via 2000, Schoonhoven 2005) where different questions are still under research in relation with the evolution of specialization, their association with speciation, and circumstances promoting that specialized populations can diverge to the extent that they can become into separate species (Futuyma & Moreno 1988, Caillaud & Via 2000). Taking this into account, it becomes an important task for researchers to investigate about characters involved in specialized habitat or resource use.

The existence of trade-offs on different host plants has been a central hypothesis trying to explain evolutionary specialization (Futuyma & Moreno 1988, Jaenike 1990, Agrawal 2000). Considering that different habitats (e.g. host plants) constitute potentially different selective scenarios, phytophagous insects using alternative host plants must reflect the effect of environmental differences on traits associated with performance like, life cycle duration, mortality, signalling and perception systems (Futuyma & Moreno 1988, Jaenike 1990, Agrawal 2000, Nosil *et al.* 2007). Nevertheless, evidence about costs or benefits associated to the use of alternative hosts is still not conclusive, since even when in a few cases trade offs have been identified, the mechanisms causing them still remain unidentified (Jaenike 1990, Agrawal 2000, 2002, Schoonhoven 2005, Rios *et al.* 2013).

The ecology and evolution of host range for phytophagous insects could be better understood if host preference and subsequent performance on alternative plants are studied (Agrawal 2000). Performance could be addressed from a population point of view taking into account that demographic variables should show low amplitude and stable population dynamics if an insect prefer a host plant over other species to mate, oviposite or feed, and having this preference a positive association with their performance (Jaenike 1990, 1996, Price *et al.* 1990, Price 1994, Caswell 2001). Since, at population level the instantaneous growth rate (λ), can work as an average fitness proxy, the estimation of this demographic variable in individuals of the same insect species developing in two different hosts, must

show the effect of biotic + abiotic environmental variables, is a consequence of changes in fecundity, survivorship and developmental rates (Charlesworth 1980, Caswell 1983, 2001, Larsson *et al.* 2000, Awmack & Leather 2002). Thus, the reproductive output should vary with the host plant where insects develop. Nevertheless, empirical evidence about the interaction between Host plant – Insect performance/demography in systems where alternative hosts plants are used is scarce (Larsson *et al.* 2000, García-Robledo & Horvitz 2011, Rios *et al.* 2013).

Treehoppers (Hemiptera: Membracidae) constitute a c.a. 3200 sp. group of sap feeding insects depending entirely of their host plant for subsistence, performing on them most of their ecological and biological processes (e.g. feeding, mating, oviposition, development). Thus, the effect of this dependence is reflected in various genera of treehoppers showing host specialization where a synchrony between the insect's life cycle and host-plant phenology is observed (Wood & Olmstead 1984, Wood 1993b, Sattman & Cocroft 2003). On the other hand, social patterns are also quite variable on treehoppers, ranging from the absence of sociality in species exhibiting solitary modes of life, until species with nymphal or adult aggregations which displays a set of behaviors characteristic of the subsocial level (Wood 1993a, Stegmann & Linsenmair 2002, Cryan *et al.* 2004, Lin 2006), defined as a group of relatives consisting of one or both parents and their immature offspring, characterized by brood defense or brood provisioning by one or both parents (Crespi & Yanega 1995, Linksvayer 2010). Those behaviors in treehoppers

are linked to maternal care and therefore play a key role in survival of developing individuals (Tallamy & Schaefer 1997), which in turn is a relevant fitness component of insects that posses social tendencies or belong to some social category like subsocial, communal, quasisocial, semisocial and eusocial (Wilson 1971, Crespi & Yanega 1995; Linksvayer 2010). Taking into account the relevance of parental care as a behavioural attribute directly involved in the colony (or group) maintenance and productivity, we propose that this behavioural pattern is an attribute that may variate according to the host plant used as habitat and/or resource. To our knowledge, variations in social behaviours and it`s consequences in performance and demography have not been evaluated in treehoppers where alternative hosts plants are used. *Alchisme grossa* (Membracidae, Hoplophorionini) is highly distributed in neotropics, belonging to a tribe where all the members display sub social patterns (maternal care + nymphal aggregation) as an ancient trait, which include behaviors like antidepredatory defense, egg guarding and nymphal facilitation at different extents (Wood 1993a, 1993b, McKamey & Deitz 1996, Lin *et al.* 2004, Camacho *et al.* 2013, Torrico-Bazoberry *et al.* 2014). In general, the genus *Alchisme* use Solanaceus plants as hosts, fact that is suggested to be linked directly with plant chemistry (Wood 1993a, McKamey & Deitz 1996, Pinto, unpublished data). Suporting this pattern, *A. grossa* utilizes *Brugmansia suaveolens* (Humb. & Bonpl. ex Wild) Bercht. & C. Presl (Solanaceae) and *Solanum ursinum* (Rusby) (Solanaceae) as host plants growing in sympatry in cloud forests of Bolivian Yungas (Torrico-Bazoberry *et al.* 2014), where females oviposite and

care nymphs along their development, besides, groups of *A. grossa* living on each host, showed patterns of dispersion who only includes the host where they born (Pinto, unpublished data).

Using *A. grossa* – Host plant association as models, we investigate how these two hosts can act as differential environments promoting changes in demographical parameters of groups of insects living on them in natural conditions. We also characterize performance of families (mother+ nymphs) on both hosts including behavioral traits related to subsociality.

3.3 Materials and Methods

Study area and data collection

Since, *A. grossa* can be found on both of its host plants during the rainy season (Torrico-Bazoberry *et al.* 2014), data were collected from December 2013 to April 2014 at Incachaca locality (Cochabamba, Bolivia, 17º13'S - 65º49'W; 2450 m.a.s.l.), within the Yungas biogeographical region. We worked in a zone with plants of *B. suaveolens* and *S. ursinum* coexisting in simpatry, where also a considerable number of families of *A. grossa* (i.e. adult female with egg mass) exist. In the first trip, at the beginning of rainy season, each plant was tagged with a sequential plant code, and also, petioles of leaves containing families were tagged with a code including a letter for the host-plant and a number for the family. Adult females/mothers on each family were momentary removed to measure length and

width of egg masses with a Goldtool® digital caliper (0.01 mm. precision) to estimate the number of eggs present on the mass following Torrico-Bazoberry (unpublished data). Pronotum of mothers were also marked on the right side with a permanent marker (Stabilo) with the same code used in the petiole of the leaf and placed back over its respective egg mass. This process was performed only one time in order to count with families (mothers + eggs) of approximately the same age to be followed along all their development during subsequent trips.

Life cycle duration and families following on alternative hosts

To characterize life cycle parameters of *A. grossa*, all tagged families on plants were monitored once every 4 days (between 11:00 and 14:00 h) following Torrico-Bazoberry *et al.* 2014. On each observation the following parameters were registered at family level: a) number and development stage (instar) of nymphs, b) local abiotic variables (temperature, luminosity and relative humidity), microenvironmental traits on these leaves (light in lux, temperature in °C and relative humidity in %) were characterized, using a light meter (Lutron ® LX-101) and an thermocouple (EXTECH®) positioned 2 cm. below the underside of leaves on each family, and c) number of parasite mites on mothers and nymphs.

At plant level, we characterize: a) Richness and abundance of predators on each data collection along families development. b) Also, a herbivory index was calculated following Domínguez & Dirzo (1995) using data obtained on a single day

on February (midway in the study period) on 10 randomly chosen leaves of each marked plant. This index was considered an indirect measure of competition between *A. grossa* and other phytophagous insects (i.e. folivores). Finally, c) to investigate if host plants represent different microenvironmental refuges to *A. grossa,* the macroenvironmental temperature and relative humidity of the study location were compared with the microenvironmental data of each host-plant species using data of a HOBO climatic station based on the diurnal time interval (6:15 am – 18:15 pm, representing the 12 hours interval of diurnal values). Data acquisition at family and plant level was performed until all families completed development or disappeared.

Total life cycle duration (i.e. duration in days since the egg mass was marked until offspring reach the adult stage) of those families that successfully completed development was compared between host-plant species using a t test. Also, the duration of each developmental stage was compared between host-plant species using a Mann-Whitney test. The abiotic microenvironment of each family was characterized by the mean value of temperature, relative humidity and luminosity using data registered at each observation period (one time every 4 days). Comparisons of environmental parameters between families on *B. suaveolens* and on *S. ursinum* were performed using a Mann-Whitney test. Registers from the climatic station, and an average value for each host-plant species (estimated as the mean value of each individual plant, which in turn was estimated as the mean value

using data of each family present on the plant) of each monitoring date were used as replicates and compared using t test analyses with the two host-plants species analyzed separately (i.e. climatic station data vs. *S. ursinum* and climatic station data vs. *B. suaveolens*). Finally, Biotic environment of marked families was characterized by: i) the abundance and diversity of predators per plant during the observation period, jointly expressed as the Shannon-Wiener index, ii) the mean number of parasitic mites per family (i.e. on the female and/or the nymphs) observed during all the observation periods, and iii) the herbivory index per plant. Comparisons between host-plant species were performed with Mann-Whitney tests for all parameters except for herbivory index, where a *t*-test was used (Sokal & Rohlf 1995).

Insect performance on host-plants

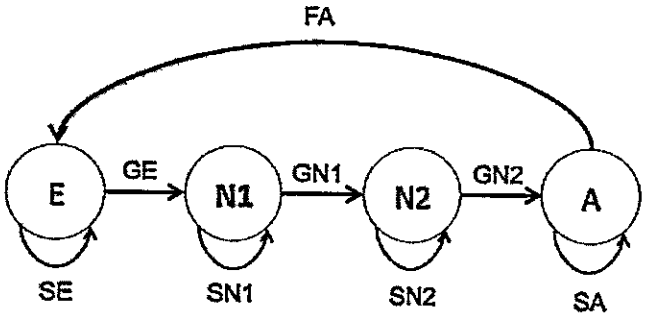
To assess performance of *A. grossa* living on each host plant species, the following comparisons were carried out between host-plant species considering each individual plant as a replicate: a) the proportion of developed families on each plant (i.e. number of families whose nymphs completed development / number of initial marked families), b) the total number of adult offspring per initial families on each plant (i.e. total number of adult offspring counted per plant / initial number of reproductive females marked per plant) and c) the total number of adult offspring per final families on each plant (i.e. total number of adult offspring / number of families whose complete development on each plant). Comparisons were

performed using Mann-Whitney tests, except for the latter that was compared using a t test on SigmaPlot 12.0.

Demographic analysis

Life cycle of *A. grossa* involves seven stages: egg, 5 nymphal instars and imago However, based on biological and ecological observations that first and second nymphal instar are not mobile and depend completely on maternal care for survival (Torrico- Bazoberry *et al.,* 2014), they were comprised as a single stage for modelling population dynamics. At the same time, third, fourth and fifth nymphal instars were comprised as a single stage since they are very mobile stages that do not depend completely on maternal care for survival (Torrico-Bazoberry *et al.* 2014). Thus, life cycle of *A. grossa* was modelled as a 4 stage matrix: egg (E), nymph 1 (N1; which comprises first and second nymphal instars), nymph 2 (N2; which comprises third, fourth and fifth nymphal instars) and adult (A) (Figure 2A).

A)



B)

$$A = \begin{bmatrix} SE & 0 & 0 & FA \\ GE & SN1 & 0 & 0 \\ 0 & GN1 & SN2 & 0 \\ 0 & 0 & GN2 & SA \end{bmatrix}$$

Figure 2. *Alchisme grossa* stage-classified life cycle. **(A)** Graphic representation: E=eggs, N1=nymph 1 (first and second nymphal instars), N2=nymph 2 (third, fourth and fifth nymphal instars) and A=adults. **(B)** demographic matrix based on four-stage life cycle: SE = probability to survive and remain as egg, GE = probability to survive and reach the next stage for eggs, SN1 = probability to survive and remain as nymph 1, GN1 = probability to survive and reach the next stage for nymph 1, SN2 = probability to survive and remain as nymph 2, GN2 = probability to survive and reach the next stage for nymph2, SA = probability to survive and remain as adult, and FA= adult fecundity.

Female proportion was determined for *A. grossa* adults on each host plant (0.56 for females on *B. suaveolens* and 0.54 for females on *S. ursinum*) (Pinto, unpublished data) in order to work only with the female component of the population as is suggested by Caswell 2001. Then, the number of eggs, nymph 1, nymph 2 and adults on each host plant was multiplied per female proportion respectively. Nymphs of *A. grossa* of the last nymphal instars form mixed families aggregations within a single plant but they are limited to move within host-plants (i.e. completing its life cycle on the host-plant where they were born) (Torrico-Bazoberry *et al.* 2014), thus a total of 29 four-stage demographic matrixes were constructed (one for each host-plant, 13 for *B. suaveolens* and 16 for *S. ursinum*) comprising data of all families marked on each host plant. These matrixes included the following demographic parameters: fecundity of adults (FA), the probabilities of surviving and remaining at the same stage (stasis) for an egg, nymph 1, nymph 2 and adult (SE, SN1, SN2 and SA, respectively); and the probabilities of surviving and reach the next stage (growth) for an egg, nymph 1 and nymph 2 (GE, GN1 and GN2, respectively) (Figure 1A). Adult fecundity (FA) was determined as the average number of eggs deposited by the females ovipositing on each individual host plant. Other demographic variables (S_i and G_i) were estimated following Caswell (2001). Adult (i.e. female) fecundity was compared between host-plants using a t test.

An individual matrix model was constructed for each plant (Figure 2B); where two analyses were conducted, the first one to obtain the finite rate of increase of the population (λ), calculated as the dominant eigenvalue of the matrix (Caswell 2001,

Hood 2010), and the second one to obtain the elasticity matrix which includes the proportional contribution of each demographic parameter to variations in λ (Caswell 2001). These analyses were conducted using Poptools (Hood 2010).

A mean λ value and a mean elasticity matrix were obtained for *A. grossa* ovipositing on *B. suaveolens* (n=13) and those ovipositing on *S. ursinum* (n=16) using each plant as a replicate. Also, in order to determine which type (G, S or F) of the demographic parameters contributed more to changes in λ at an intra-host level, two one way ANOVA comparisons followed by a Holm-Sidak *post-hoc* test were performed considering the type of demographic parameter (fecundity, stasis or growth) as a factor, each matrix as a replicate, and using the total sum of each parameter of each type of the matrix as the dependant variable (i.e. stasis = SE+SN1+SN2+SA, growth = GE+GN1+GN2, fecundity = FA) (Pfister 1998). T test and Mann-Whitney comparisons of each demographic parameter were also performed between host-plant species, in order to assess the input to λ by stasis, fecundity and growth. All statistical analyses were performed on SigmaPlot 12.0.

Also, to evaluate if λ values could be explained by any of the abiotic microenvironmental (i.e. temperature, relative humidity and light) or biotic microenvironmental [i.e. predator diversity (Shannon-Wiener index), parasitism (number of parasitic mites per family) or indirect competition (herbivory index)] variables at the plant level, 12 general linear models (GLM) were performed

considering λ as the dependent variable and one of the environmental variables as the predictor variable for each host-plant species separately. GLM were performed on Statistica 10.0.

Sociability

Subsociality is characteristic in Hoplophorionini treehoppers, involving the evolution of maternal care of developing individuals (eggs and nymphs) which are in aggregation, being a behavioural pattern that include tasks like egg and nymphal guarding, antidepredatory defense and feeding facilitation (McKamey & Deitz 1996; Lin *et al.* 2004; Torrico-Bazoberry *et al.* 2014). For *A. grossa*, duration of maternal care for each marked female was estimated as the total number of days that female was observed next to its offspring performing this task. Then, Spearman correlation tests were performed in groups of *A. grossa* living in each host plant to assess the relation between the duration of maternal care, as a proxy of subsociality, and the number of nymphs belonging to a particular instar, as proxy of offspring survival, in three immature instars: i) the number of hatched nymphs (i.e. number of first instar nymphs per female), ii) the number of second instar nymphs per female and iii) the number of third instar nymphs per female. Also, duration of maternal care was compared between host-plant species using Mann-Whitney tests: a) considering the total duration of maternal care (as described above) and b) considering duration of maternal care minus duration observed at the egg stage, thus taking in to account only maternal care duration after eggs hatched. The latter

comparison was performed since total duration of maternal care could be biased by the development stage that the egg mass had when the family was marked. Correlation analyses were also repeated taking in consideration the absence of egg mass instar duration, in order to evaluate if patterns were maintained. All tests were performed on SigmaPlot 12.0.

3.4 Results

A total of 120 families were marked during the season (69 on *B. suaveolens* and 51 on *S. ursinum*). Total life cycle duration of those families that completed development differed between host-plant species (t=3.08; d.f. = 38; p=0.00383), being longer on *B. suaveolens* (Table 5). Particularly, duration of egg stage and first nymphal instar differed between host-plant species (U=103.5; p=0.01 and U=124.5; p=0.043 respectively), both being longer on *B. suaveolens.*

Table 5. Life cycle duration of *Alchisme grossa* on *B. suaveolens* and on *S. ursinum.* * shows statistical difference between host-plant species at p<0.05

Stage	Mean ± SD duration on days on *B. suaveolens*	Mean ± SD duration on days on *S. ursinum*
Egg*	33.4 ± 5.5	25.3 ± 10.8
First instar*	11.8 ± 3.0	9.7 ± 3.3
Second instar	10.4 ± 3.3	9.1 ± 3.8
Third instar	12.0 ± 4.5	10.5 ± 3.9
Fourth instar	15.2 ± 4.0	16.0 ± 3.9
Fifth instar	24.7 ± 7.6	24.1 ± 5.0
Total life cycle*	107.5 ± 12.3	94.8 ± 13.7

Percentage of relative humidity measured with the climatic station and that of the host-plant species did not differ, however a marginal significant difference ($p=0.0543$) was observed between the climatic station (85.023 ± 5.602 %) and *S. ursinum* (79.945 ± 6.597 %). Temperature on *B. suaveolens* (19.063 ± 1.934 °C) and *S. ursinum* (20.064 ± 2.014 °C) did significantly differ ($t=-3.223$; d.f.=22; $p<0.01$ and $t=-4.150$; d.f.=22; $p<0.001$ respectively) with the climatic station (16.792 ± 1.543 °C). These results indicate that both host-plants could act as micro environmental refuges for *A. grossa*, generating a higher temperature environment than that of the exterior (macroenvironment).

Abiotic (luminosity, temperature and relative humidity), and biotic (abundance and diversity of predators per plant and herbivory index) traits were statistically different between host-plants species (Table 6), whereas mean abundance of mites per family did not differ. Mean luminosity and temperature values were higher on *S. ursinum* ($p<0.05$ and $p<0.001$ respectively) while relative humidity was higher on *B. suaveolens* ($p< 0.001$). Herbivory index was also higher on *S. ursinum* ($p< 0.001$), suggesting a possible higher competition between *A. grossa* and other phytophagous insects. A total of eight predator morphospecies were registered on both host-plants: four species of spiders (Aranae), and four insect species; an assassin bug (Hemiptera; Reduviidae), a lady beetle (Coleoptera; Coccinellidae), an elaterid (Coleoptera; Elateridae; *Semiotus sp.*) and a wasp (Hymenoptera; Vespidae). None of these species were exclusive to a given host-plant; however,

predator diversity and abundance were higher on *B. suaveolens* ($p<0.05$ and $p< 0.001$ respectively, Table 2) showing that families that develop on this host-plant are more exposed to predation than those that develop on *S. ursinum.*

Table 6. Mean values ± SD and comparison of abiotic and biotic environment between *B. suaveolens* and *S. ursinum*. Predator diversity is represented as the Shannon-Wiener index.

Parameter	*B. suaveolens*	*S. ursinum*	Statistical	*p*
Luminosity (lux)	85.32 ± 54.35	131.52 ± 97.59	$U = 1297$	0.014
Temperature (°C)	18.96 ± 1.18	20.45 ± 2.15	$U = 946.5$	< 0.001
Relative humidity (%)	82.90 ± 4.60	77.89 ± 6.19	$U = 917.5$	< 0.001
Predator abundance per plant	3.50 ± 3.70	0.71 ± 1.25	$U = 225.5$	< 0.001
Predator diversity per plant	0.32 ± 0.24	0.12 ± 0.17	$U = 66.5$	0.017
Parasitic mites per family	0.78 ± 1.42	2.92 ± 8.24	$U = 1632.5$	0.444
Herbivory index (*HI*)	2.21 ± 0.74	3.27 ± 0.57	$t = -5.086$	< 0.001

There is no significant difference between the proportions of developed families per plant between host plants, however this value is on average slightly higher on *S. ursinum* (0.897 ± 0.231) than on *B. suaveolens* (0.721 ± 0.338). Also, there is no significant difference in any of the other performance variables considered in this study for comparisons between host plants like the total number of adult offspring per initial families in each plant (i.e. total number of adult offspring / initial number of marked females) (U=89.500; p=0.539) or the total number of adult offspring per final families in each plant (i.e. total number of adult offspring / number of families whose offspring survived to produce adult offspring) (t=-0.177; d.f.=27; P=0.861)

Adult fecundity (number of eggs) did not differ between host-plants (t=-1.653; d.f.=27; p=0.11). Finite rates of increase (λ) were lower than 1 on all *B. suaveolens* replicates (13) (0.7835 ± 0.1598) and for 15 (out of 16) of *S. ursinum* (0.7522 ± 0.1656) individuals, indicating a tendency of *A. grossa* population to decrease on both hosts. There is no statistical difference on λ between host-plant species (t=0.513; d.f.=27; p=0.612).

The proportional contribution (elasticity) of each demographic parameter to variations in λ on the average elasticity matrix is shown in Table 7. The parameter that contributed the most to variations in λ was stasis of nymph 2 on both host-plant species (SN2, 49.86 ± 38.76 % on *B. suaveolens* and 41.36 ± 40.47 % on *S. ursinum*). The contribution of the type of demographic parameters to λ also differed on both host-plant species (H= 26.759; d.f.=2; p<0.001 for *B. suaveolens* and H=34.383; d.f.=2; p<0.001 for *S. ursinum*) being the contribution of stasis higher and different of those of fecundity and growth on both host-plant species (p <0.001).

Table 7. Elasticities (Mean ± SD) of the demographic parameters of A (demographic matrix based on four-stage life cycle of *A. grossa*) on two host-plants species.

Demographic parameter	*B. suaveolens*	*S. ursinum*
FA	0.0145 ± 0.0132	0.0200 ± 0.0148
SE	0.3250 ± 0.4117	0.2970 ± 0.3428
SN1	0.1200 ± 0.1596	0.2097 ± 0.3063
SN2	0.4968 ± 0.3876	0.4136 ± 0.4047
SA	0.0001 ± 0.0001	0.0001 ± 0.0001
GE	0.0150 ± 0.0132	0.0200 ± 0.0148
GN1	0.0145 ± 0.0132	0.0200 ± 0.0148
GN2	0.0145 ± 0.0132	0.200 ± 0.0148

None of the 12 GLM analyses were statistically significant ($p > 0.05$), which suggest that any of the environmental variables considered in this work explains λ values.

Sociability

On *B. suaveolens* (BS) and *S. ursinum* (SU) strong significant correlations between maternal care duration and the number of: i) hatched nymphs (BS $r=0.786$; $p<0.01$; $n=69$ and SU $r=0.451$; $p<0.01$; $n=51$), ii) second instar nymphs (BS $r=0.778$; $p<0.01$; $n= 69$ and SU $r=0.569$; $p<0.01$; $n=51$) and iii) third instar nymphs (BS 0.725; $p<0.01$; $n=69$ and SU $r=0.488$; $p < 0.01$; $n=51$) were observed. Identical patterns in significance were maintained after repeat analysis using maternal care duration after egg hatching on both host plants species, in fact correlation coefficient (r)

incremented in all cases: i) hatched nymphs (BS r=0.790; p<0.01; n=69 and SU r=0.577; p<0.01; n=51), ii) second instar nymphs (BS r=0.865; p<0.01; n= 69 and SU r=0.663; p<0.01; n=51) and iii) third instar nymphs (BS 0.821; p<0.01; n=69 and SU r=0.599; p < 0.01; n=51) respectively. There are no significant differences in the duration of maternal care between host plant species, neither considering the total duration (U= 1393; p= 0.051) nor considering data after egg hatching (U= 1617; p=0.423). Maternal care was extended at least along the first three nymphal stages on both host plants (100 %). Nevertheless, after this point, nymphs form mixed aggregations in the main stem of them, and practically all mothers abandoned the plant on both hosts.

3.5 Discussion

Cohorts of *A. grossa* growing in *B. suaveolens* and *S. ursinum*, differ in the mean duration of life cycle, being longer on *B. suaveolens.* Both host plants showed a higher temperature than the environment, besides, abiotic and biotic traits measured at microenvironmental level on each host showed differences between them, which demonstrate that both host plants are quite different as developmental scenarios. Nevertheless, performance of *A. grossa* did not differ between host plants, a fact that is also supported by the absence of differences in demographic parameters compared between both hosts. Lambdas on both hosts showed values under 1, where stasis constitutes the parameter that contributes more to changes in lambda. Finally, maternal care traits measured, demonstrate

that females on both host plants perform this task with the same quality and intensity on both hosts.

Life cycle duration differences in *A. grossa* living on *B. suaveolens* and *S. ursinum* could be a direct product of alternative host use, involving at the same time potential effects on insect fitness, ecology and biology (Futuyma & Moreno 1988, Jaenike1990, Wood 1993a). Specifically, individuals developed on *B. suaveolens*, are exposed for longer time to different risks affecting their fitness (e.g. predation events, weather phenomena, parasites or diseases) (Wood 1993a, Wood *et al.* 1999, Bernays & Chapman 1994, Schoonhoven 2005). Besides, this difference in life cycle duration can also have an important effect in the reproductive isolation of groups living on each host plant, first because almost all individuals of *A. grossa* disperse exclusively on the same host of development (Pinto, unpublished data) and second, because *A. grossa* show natural synchrony of cohorts where almost all individuals in natural populations are in the same age or reproductive stage at the same time (Unpublished data). Other treehoppers which use alternative hosts also showed changes in traits like life cycle duration, or modification of signals involved in mate recognition interfering with a free interchange or crossing of individuals from alternative hosts (Lin & Wood 2002, Cocroft *et al.* 2008, 2010). Thus, the current ecological scenario reported in groups of *A. grossa* living in each host plant, i.e. asynchrony in developmental time + dispersion patterns, should reduce the chance of encounters in time between reproductive individuals developed on each host, a

hypothesis that require asses genetic structure between *A. grossa* living in both hosts. In addition, previous patterns described in these contexts has been associated with the onset of diversification via ecological speciation, where host-specialized species occur via disruptive selection on host preference with the feedback of the preference of organisms to mate on their favorite host (Futuyma & Moreno 1988, Wood *et al.* 1999).

Both hosts seems to constitute a refuge for treehoppers since they offer protection from environmental conditions (e.g. warmer temperature on leaves selected by mothers to oviposite), nevertheless, both biotic and abiotic microenvironmental traits characterized on each host are quite different, suggesting that *B. suaveolens* and *S. ursinum* are totally distinct scenarios for *A. grossa* development. Quantity of sun light and relative humidity could be directly associated with temperature, where thermal increase in breeding sites of ectothermic organisms also increase the rate of development, reducing the total time to reach maturity (Charnov & Gillooly 2003, Kingsolver & Huey 2008) favoring the fast development rate *of A. grossa* on *S. ursinum*. However, effects of plant chemistry such as alkaloids present along all the Solanaceae family cannot be discarded. For example, in the case of *B. suaveolens* scopolamine has been characterized as responsible to increase life cycle duration in other specialized insects feeding on them (Alves *et al.* 2007). On the other hand, biotic parameters characterized for each host also suggest that *B. suaveolens* represent the suboptimal host, since diversity and abundance of *A. grossa* predators is around 4 times compared to those reported on *S. ursinum*. In

relation with this, potential factors counteracting these costs for *A. grossa* living on *B. suaveolens* should exist: e.g. more competition for resources on *S. ursinum* where herbivory indexes are higher, or better nutritional quality of *B. suaveolens* respect to *S. ursinum*, or the escape of natural predators/parasitoids in the opposite direction (Berdegue *et al.* 1996, de-Silva *et al.* 2011) and/or the capability of *A. grossa* to sequester tropanic alkaloids from *B. suaveolens* (unpublished data), compounds already related with chemical defense in insects, which could be advantageous in the survival during nymphs or adult stages (Schoonhoven 2005, Alves 2007, Pinto *et al.* 2011).

It has been proposed that host plant quality affect diverse traits of phytophagous insects living on more than one host (e.g. fecundity, survivorship) causing finally variations in the reproductive output on each host, and also transferring this effect into population dynamics of individuals developing on them, nevertheless, research about this interactions in natural condition,s only count with a few examples (Larsson *et al.* 2000, Awmack & Leather 2002, Schoonhoven 2005, García-Robledo & Horvitz 2011). *A. grossa* did not differ in any of the measures in performance compared between individuals developing on both highly qualitatively different hosts (*S. ursinum* and *B. suaveolens*), and this is also reflected in the absence of differences in population demographic parameters. In that sense, finite rate of increase (lambda) reflects one point in time along the dynamics of a population, the last one containing also natural oscillations that can vary in their intensity between localities and species (Caswell 2001, Larsson *et al.* 2000, Awmack & Leather 2002),

this fact can in some way explain why on both hosts lambda values are mostly under one. Comparisons between inputs of demographic parameters to changes in lambda (Anova within each host comparing total value of each parameter observed in elasticity matrixes) showed the same pattern on both hosts, being stasis the most important one, this support an apparent consistent pattern, where diverse taxa (vertebrates, invertebrates, plants) showed that the bigger input came from stasis (survivorship) followed by growth and fecundity (Caswell 2001, Enright *et al.* 1995, Pfister 1998). Both performance and population dynamics are showing that genotypes of *A. grossa* developing on *B. suaveolens* or *S. ursinum* achieve a balance on their respective hosts overcoming potential trade offs associated with development on alternative plants.

Hoplophorionini species present the highest degree of maternal investment among treehoppers (McKamey & Deitz 1996), especially since maternal care, an ancient trait evolved in the tribe (Lin *et al.* 2004) has probably evolved due to the absence of mutualistic interactions with ants or other hymenopterans (McKamey & Deitz 1996, Lin *et al.* 2004). Maternal care has been probed to be crucial to offspring survival in Hoplophorinini species, as has been showed by Cocroft (2002) in *Umbonia crassicornis*, where the absence of females leads to a four-times increase in predator success in offspring removal, or the case showed by Torrico-Bazoberry *et al.* (2014) for *A. grossa* where female removal lead to a 100 % of offspring mortality. In this study, we found a positive and significant correlation between maternal care duration and the number of nymphs at three different stages which

reinforce the idea that maternal protection, evidenced by mothers remaining on or near the egg masses for some period following oviposition, or actively defending their progeny, may have evolved as a parsimonious means of decreasing offspring mortality (Tallamy & Schaefer 1997). At the same time, the absence of differences on maternal care duration between both host plants, strongly suggest that this social trait can act as one of the key factors promoting the use of alternative hosts in *A. grossa*, through the equilibration of costs of traits that can work against offspring performance between hosts (e.g. antidepredatory defense, feeding facilitation).

4. CAPITULO 3: SEQUESTRATION OF TROPANE ALKALOIDS FROM *Brugmansia suaveolens* (Solanaceae) BY THE TREEHOPPER *Alchisme grossa* (Hemiptera: Membracidae) IN THE BOLIVIAN YUNGAS FORESTS

4.1 Abstract

Treehoppers (Hemiptera: Membracidae) are sap-feeding insects distributed mainly in tropical regions. *Alchisme grossa* is a treehopper that has been reported in the Bolivian Yungas forests using only two species of the Solanaceae as host-plants, *Brugmansia suaveolens* and *Solanum ùrsinum*. Adult females of *A. grossa* remain on the host-plant where they oviposit and take care of their nymphs until they molt to adults. *Brugmansia* is a subtropical genus producing a variety of tropane alkaloids (TAs). We herein report the sequestration of TAs by adult males and females of *A. grossa* from *B. suaveolens*, its preferred host-plant throughout the year, examining separately the distinct body sections of insects in order to look for possible differential sequestration. Purified extracts of *A. grossa* and *B. suaveolens* were analyzed by gas chromatography / mass spectrometry. TAs found in *A. grossa* were the same as those in its host-plant; they were differentially allocated within the body of adult individuals. The presence of TAs mainly in the pronotum of *A. grossa* reinforces its role as a defensive structure in a new chemical context. However, the overall effect of TAs on this phytophagous species may depend on its level of ecological specialization.

4.2 Introduction

A conspicuous and morphologically variable pronotum constitutes the characteristic trait of treehoppers (Hemiptera: Membracidae), a diverse group (ca. 3300 spp.) of phytophagous insects distributed mainly in tropical regions of the world (Funkhouser 1917, Wood 1993, Dietrich *et al.* 2001, Lin 2006); this structure has been assigned a role in crypsis and defense (Wood 1974, 1993, Godoy *et al.* 2006, Roy *et al.* 2007). Treehoppers also show a variety of social levels, with some species exhibiting a solitary mode of life, others including the formation of groups of adults and/or nymphs, and still others showing maternal care and nymphal aggregations, i.e. subsocial features (Godoy *et al.* 2006). Mutualistic associations with hymenopterans (e.g. ants, bees) have also been observed in some species of this group; this interaction involves obtaining the protection of nymphs in exchange for honeydew produced by nymphs and adults (Godoy *et al.* 2006).

Treehoppers are sap feeders (phloem and/or xylem), and their host-plants constitute a site for reproduction, oviposition and development (Wood *et al.* 1999, García-Robledo & Horvitz 2012). Species range from monophagous to polyphagous (Dietrich & Deitz 1991, Wood 1993, Cryan *et al.* 2004, Morales & Beal 2006, Lin 2006, 2007); diet breadth has been related to latitude, where tropical species tend to be oligophagous or polyphagous and temperate species tend to be monophagous, and to altitude, higher elevations seeming to promote the process of host specialization (Wood & Olmstead 1984, Wood 1984, Reithel & Campbell 2008).

Treehoppers of the Hoplophorionini tribe are among the most specious groups among the New World treehoppers. They are restricted mostly to montane or submontane habitats in tropical latitudes, where ecological specialization in host-plant use and preference for specific climatic ranges may promote their high diversity (McKamey & Deitz 1996). While mutualistic interactions are absent in all members of this tribe, complex behavioral traits related to maternal care are present in all of them (Wood 1974, McKamey & Deitz 1996, Camacho et al. 2013). The genus *Alchisme* (Hoplophorionini) thrives in humid montane and submontane ecosystems from northern Central America to the Brazilian shield and northern Chile, and shows distinct preference for solanaceous host-plants (McKamey & Deitz 1996, Godoy *et al.* 2006).

Alchisme grossa is a neotropically distributed subsocial species that displays diverse maternal care traits such as: egg and nymphal guarding, active defense against predators, and feeding facilitation (Godoy *et al.* 2006, Camacho *et al.* 2013, Torrico-Bazoberry *et al.* 2014). Adult females of *A. grossa* remain on the host-plant where they oviposit and take care of their nymphs until they molt to adults (Bazoberry et al. 2014). In the Bolivian Yungas forests, it uses only two species of the Solanaceae as host-plants, *Brugmansia suaveolens* and *Solanum ursinum* (Torrico-Bazoberry *et al.* 2014), in spite of this being a habitat characterized as a site of high diversity of solanaceous species (Nee *et al.* 2007). Both males and

females show host fidelity to the plant species where they were born, and the preferred feeding sites of *A. grossa* are young leaves of *B. suaveolens* (Torrico-Bazoberry *et al.* 2014).

The Solanaceae is a family comprising around 3500 species (D'Arcy 1986, Knapp *et al.* 2004), many of which contain large quantities and varieties of alkaloids (Evans 1986, Hawkes *et al.* 2000, Cordell *et al.* 2001). In several insect species, specialized mechanisms related with the use of plant secondary metabolites such as alkaloids have evolved in distinct ecological contexts (e.g. defense, host specialization) (Opitz 2009, Arab *et al.* 2012). *Brugmansia* is a subtropical genus producing a variety of tropane alkaloids (TAs) (Doncheva *et al.* 2006) which are mostly concentrated in young leaves, flowers and unripe fruits, all of them tissues highly related with plant fitness (e.g. photosynthesis and reproduction) and hence likely to be involved in processes such as chemical defense against herbivory (Alves *et al.* 2007, Arab *et al.* 2012). In this study, we investigate the sequestration of TAs from *B. suaveolens* by adult males and females of *A. grossa*, examining separately the distinct body sections of insects (pronotum, head+thorax+abdomen, and wings+legs) in order to look for possible differential sequestration patterns related with their different ecological roles.

4.3 Materials and methods

Alchisme grossa and *Brugmansia suaveolens*

Field work was performed at Incachaca (Cochabamba – Bolivia: 17°13S, 65°49W; 2450 m.a.s.l.) within the Yungas biogeographical region during February and March 2013. In this area, *A. grossa* females oviposit mostly on young leaves of *B. suaveolens*, their preferred host-plant throughout the year (Torrico-Bazoberry *et al.* 2014). Periodic observations showed that cohorts were synchronized, allowing collection of ten males and ten females from different cohorts within two days of having reached the adult stage. Immediately after collection, insects were taken to the laboratory, transferred to tomato plants (*Lycopersicum esculentum*) enclosed in tulle bags and allowed to feed for 24 h. During this time the insect replaces *B. suaveolens*-related contents in its gut by tomato-related contents; thus, TAs found in *A. grossa* correspond only to compounds sequestered in the insect body.

Young leaves (N= 4 groups) were collected from different individuals of *B. suaveolens* where insects were found, to identify and quantitate TAs in them. Plant samples were carried to the laboratory, dried at 35°C (Heraeus UT6 oven) for three days, ground in a laboratory mill, and stored until chemical analyses were performed.

Extraction of alkaloids from insects

Insects were sacrificed by freezing and then dried at 35º C (Heraeus UT6 oven) for three days. Each adult was weighed (8.06 ± 1.19 mg dry weight; mean ± SD) and its body dissected under a stereoscopic magnifying lens (Olympus SZ61) into three sections: a) pronotum (18.7 ± 0.024 % d.w.), b) head+thorax+abdomen (66.3 ± 0.083 % d.w.), and c) wings+legs (15.0 ± 0.028 % d.w.). Each section was ground separately by introducing it into a 1.8 ml stainless steel microvial with a polyethylene flange cap containing five 1.5-mm-diameter steel spheres and agitating it for 10 min in a bead beater (Mini-Beadbeater-96; Biospec Inc., Bartlesville, OK, USA). The pulverized sample was extracted with 2 ml CH_3OH and exposed to ultrasound in a bath (Power Sonic 405) at 25ºC for 30 min. The methanolic extract was filtered through cotton wool placed at the tip of a Pasteur pipette, collected in a 1.8 ml amber vial and dried under nitrogen flow. The dry residue was successively redisolved in small aliquots of methanol (ca. 50 µl) which were transferred to a 100 µl glass insert within an amber vial and evaporated to dryness by means of a nitrogen flow; this operation minimized the quantity of residue retained in the original vial walls. The dry residues were disolved in 10 µl methanol before injecting 2 µl into the GC column.

Extraction of alkaloids from leaves of *B. suaveolens*

Each pulverized sample (2 g) of *B. suaveolens* leaves was extracted with 40 ml CH_3OH and exposed to ultrasound in a bath (Power Sonic 405) at 25ºC for 30 min.

The methanolic extract was filtered through a frit funnel and the resulting extract evaporated under reduced pressure on a rotatory evaporator (Büchi RE 111). The syrupy residue was dissolved in 3 ml 5% HCl. The acidic solution was washed with $CHCl_3$ (2x3 ml). The aqueous phase was adjusted to pH 10 with NH_4OH and was extracted twice with 3 ml $CHCl_3$; under these circumstances the final extracts gave negative Dragendorff reaction. Finally, the organic extract was dried with anhydrous Na_2SO_4, filtered through cotton wool placed at the tip of a Pasteur pipette and taken to dryness by means of a nitrogen flow. Total alkaloids as free bases constituted on average of 2.1% dry weight of the plant extracts. The dry residues were disolved in 10 µl methanol before injecting 2 µl into the GC column.

Analysis of extracts by gas chromatography / mass spectrometry (GC/MS)

Purified extracts of *A. grossa* and *B. suaveolens* were analyzed by GC/MS (Shimadzu, GCMS-QS 2010 Ultra), equipped with an Rtx-5MS Crossbond 5% diphenyl - 95% dimethyl polysiloxane capillary column (30 m length, 0.25 µm film thickness, 0.25 mm internal diameter). The GC was operated in the splitless injection mode; injection volume was 1 µl for plant extracts and 5 µl for insect extracts. The column temperature was held at 30ºC for 3 min, raised at 25ºC/min to 230ºC, and maintained for 12 min at 230ºC. The carrier gas was helium at a flow rate of 1.3 ml/min. The mass spectrometer was used in the electron impact ionization mode (70 eV) with an emission current of 250 µA. The injection port, ion source and transfer line were kept at 250ºC.

Identification of alkaloids

Preliminary identification of alkaloids was achieved using the NIST 08 mass spectra library contained in the software GCMS Solution v. 2.61 (Shimadzu Corporation, Kyoto, Japan). Since this database contains few tropane alkaloids, the mass spectra and retention indexes of the chromatographic peaks were compared with those of TAs reported from *B. suaveolens* or other species of the genus *Brugmansia*. For mass spectral comparisons, similarity indexes were calculated based on the mean intensity (mean of four plant and 20 insect extracts) of the thirteen most abundant mass fragments using the algorithm in the Shimadzu software GCMS Solution v. 2.61. The following reported alkaloids were used in the comparisons: 6-hydroxyapoatropine (RI: 2042), dihydroaposcopolamine (RI: 2046), aposcopolamine (RI: 2067), 3α,6β-ditigloyloxytropane (RI: 2141), methylscopolamine (RI: 2172), scopolamine (RI: 2236), 6-hydroxyhyoscyamine (RI: 2308), 6-hydroxyapoatropine (RI: 2311), and 3,6-ditigloyloxy-7-hydroxytropane (RI: 2321) (Freitas *et al.* 1996, Doncheva *et al.* 2006).

Statistical analysis

Alkaloid concentrations were expressed as ion abundance/mg of dry tissue (IA/DW). Values were square-root (datum+1) transformed in order to be able to include zero values in the analyses and to reduce the influence of particularly high values (Quinn and Keough, 2002). Using a two-sample bootstrap test with 1000 permutations, IA/DW values for each alkaloid in each insect section (pronotum,

head+thorax+abdomen, and wings+legs) were pairwise compared between males and females of *A. grossa* individuals (Manly 2006). Differences between IA/DW for each identified alkaloid in each body section of *A. grossa* were analyzed using a permuted one-way ANOVA with 10,000 permutations followed by *post hoc* Tukey tests (Manly 2006). To explore variations in the global composition of alkaloids in insect body parts Bray-Curtis similarity indices were calculated and similarity analysis of permutations (ANOSIM) subsequently performed with 10,000 random permutations; the Bonferroni correction factor was applied to reduce type I error (Quinn & Keough 2002). To identify the compounds responsible for the variability in the differentiation between insect sections, similarities of percentages (SIMPER) analyses were performed. The statistical programs PAST, Sigmaplot 12 and the free software R (Systat Software Inc. 2008, Hammer *et al.* 2001, Dytham 2011) were used in the analyses.

4.4 Results

Alkaloids identified in *B. suaveolens* leaves

Purified alkaloidal extracts from young leaves of *B. suaveolens* contained three alkaloids with retention times of 14.5, 15.5 and 18.5 min (Fig. 3). Their mass spectra (Table 8) with a base peak at *m/z* 94 corresponding to the *N*-methylpyridinium cation and a strong peak at *m/z* 42 best represented as CH_3-$N^+{\equiv}CH$ (Blossey *et al.* 1964), suggested they were tropane akaloids. The analysis of retention and similarity indexes identified them as 3-phenylacetoxy-6,7-

epoxytropane (3-Paet), aposcopolamine (ASc) and scopolamine (Sc), respectively (Fig. 4). The molecular peaks confirmed these assignments: $[M]^+$ at *m/z* 273 ($C_{17}H_{23}NO_3$) for 3-Paet (Vitale et al., 1995), at 285 ($C_{17}H_{21}NO_4$) for ASc, and at 303 ($C_{17}H_{21}NO_4$) for Sc.

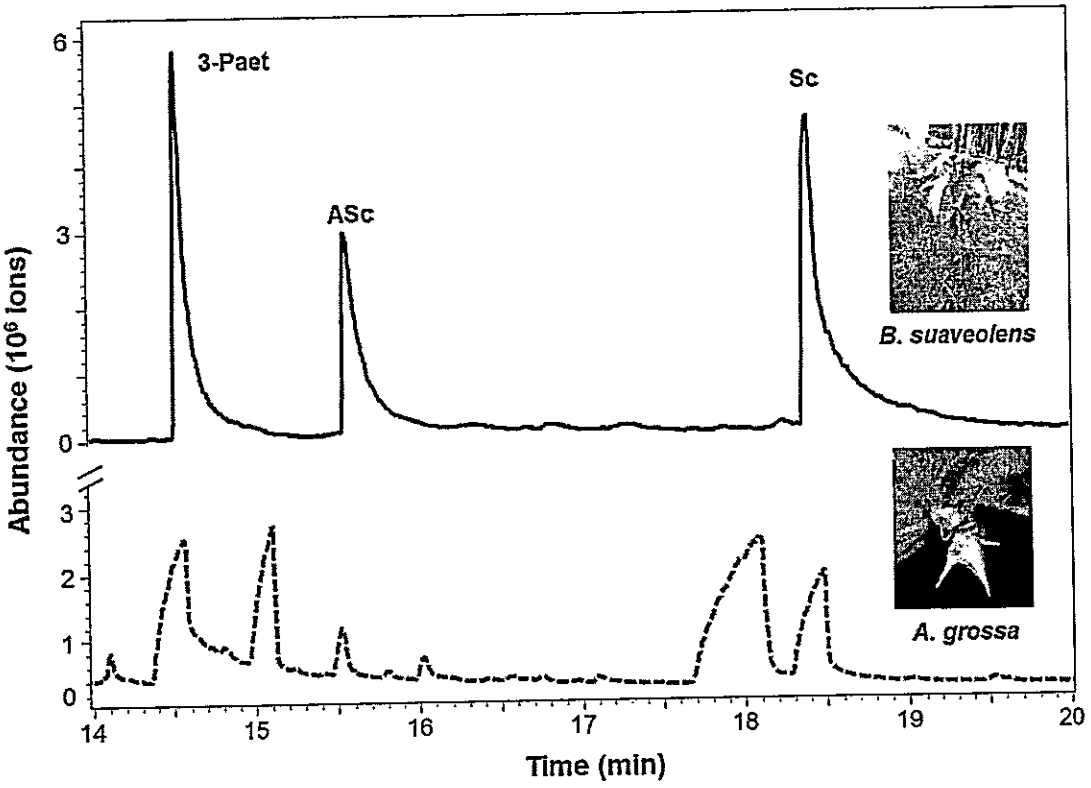
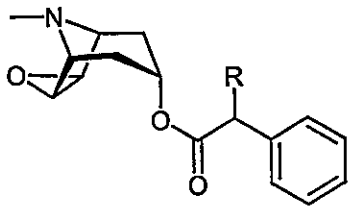


Figure 3. Chromatogram of the alkaloid fraction of: A= extracts of *B. suaveolens* young leaves and B= extracts of the pronotum of *A. grossa* showing tropane alkaloid peaks.

Table 8. Retention times (RT), retention indexes (RI) and mass spectra of alkaloids from leaf extracts of *B. suaveolens* (mean values of four extracts).

Compound	RT (min)	RI	M^+ (%)	Characteristic ions of mass spectra m/z (relative intensity, %)
1	14.5	2010	273 (21.0),	**94 (100)**, 138 (60.9), 91 (60.3), 42 (52.1), 108 (49.3), 154 (38.8), 136 (34.4), 97 (23.5), 81 (21.4), 41 (18.6), 65 (17.1), 57 (14.6)
2	15.5	2092	285 (26.3),	**94 (100)**, 103 (50.7), 138 (48.9), 42 (48.9), 108 (47.4), 154 (37.4), 136 (33.8) , 77 (26.5), 97 (23.4), 81 (20.1), 41 (15.2), 110 (17.9)
3	18.5	2248	303 (16.6)	**94 (100)**, 138 (67.8), 108 (45.9), 42 (41), 154 (35.1), 136 (34.6), 97 (22.6), 81 (20.3), 103 (18.9), 137 (16.1), 120(13.6), 110 (13.6)



3-Paet R: -H
ASc R: $=CH_2$
Sc R: $-CH_2OH$

Figure 4. Structures of tropane alkaloids found in *B. suaveolens* and *A. grossa*: 3-Paet: 3-phenylacetoxy-6,7-epoxytropane, ASc: aposcopolamine, Sc: scopolamine.

Sequestration of TAs by *A. grossa*

Freshly emerged adults males and females of *A. grossa* contained the same three TAs identified in leaves of their host plant (3-Paet, ASc, and Sc). Pairwise comparisons of IA/DW values for each alkaloid between female and male insects (pronotum, head+thorax+abdomen, and wings+legs, individually compared) showed no significant differences (P values for all comparisons were between

0.075 and 0.971). Hence, results of the analyses with insects are reported as mixed data of males and females.

Comparisons of IA/DW values between different sections of the insect body were performed for individual TAs. The pronotum showed significant differences in IA/DW values ($F= 79.96$, $d.f.= 2$, $p<0.001$), with ASc higher than 3-Paet or Sc ($p<0.05$). The same pattern (ASc > 3-Paet or Sc) was observed in the case of wings+legs ($F= 62.96$, $d.f.= 2$, $p<0.001$; post-hoc test: $p<0.05$) and of head+thorax+abdomen ($F= 54.81$, $d.f.= 2$, $p<0.001$; post hoc test: $p<0.05$) (Fig. 5).

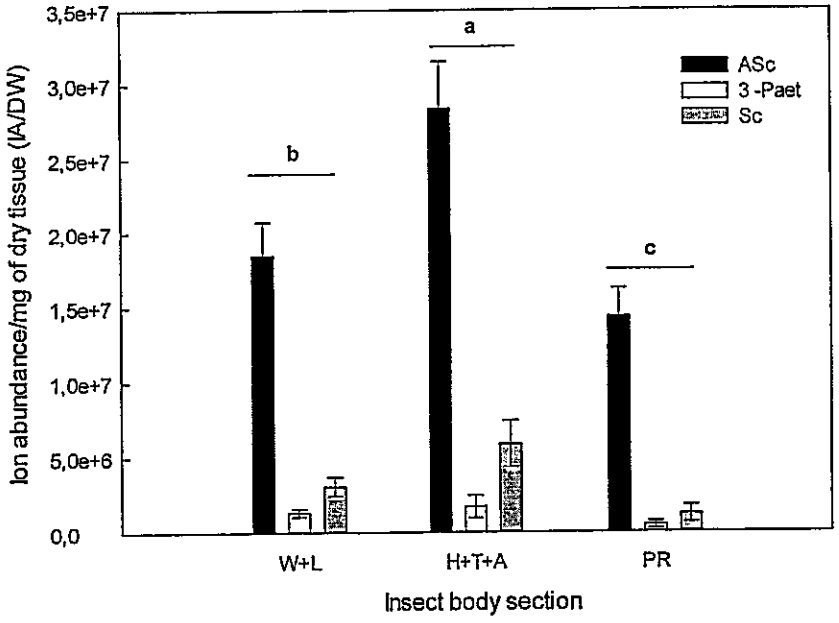


Figure 5. IA/DW values in pronotum (PR), wings+legs (W+L), and head+thorax+abdomen (H+T+A) of *A. grossa* (Mean ± SD). Letters above groups of bars denote significant differences between body parts (one way ANOSIM). ASc= aposcopolamine, 3-Paet= 3-phenylacetoxy-6,7-epoxytropane, Sc= scopolamine.

Global composition of alkaloids varied significantly between body sections (one-way ANOSIM: R=0.207, P<0.001); subsequent pairwise comparisons showed also significant differences between body sections (one-way ANOSIM: pronotum vs. wings+legs, P=0.006; pronotum vs. head+thorax+abdomen, P=0.0009; wings+legs vs. head+thorax+abdomen, P=0.021). According to the SIMPER analysis, the main alkaloid responsible for differences between body sections was ASc (42.94 % contribution).

Comparison of TAs in plant and insect tissues

Leaf extracts of *B. suaveolens* contained about 70 times more total TAs per mg of dry weight of tissue than insect tissues. In insect extracts, ASc was the most abundant TA while in plant extracts it was the least abundant. The ratios of ASc to the other two TAs were higher in insect than in plant extracts (Table 9).

Table 9. Ratio of alkaloid concentrations (IA/DW) in *B. suaveolens* and *A. grossa*. 3-Paet: 3-phenylacetoxy-6,7-epoxytropane, ASc: aposcopolamine, Sc: scopolamine.

Sample	Ratio	
	ASc/3-Paet	ASc/Sc
Plant: *B. suaveolens*	0.83 ± 0.01	0.72 ± 0.02
Insect: *A. grossa*	16.9 ± 0.2	5.1 ± 0.1

4.5 Discussion

GC-MS analysis of the alkaloidal fraction of *A. grossa* tissues revealed the presence of three TAs (3-phenylacetoxy-6,7-epoxytropane, aposcopolamine and scopolamine), all of them potentially derived from their host, *B. suaveolens,* since leaf extracts of this plant also contained these compounds (Alves *et al.* 2007). The absence of significant differences in TA concentration between male and female adults of *A. grossa* may be explained in part by a passive sequestration process, given that the feeding behavior is identical in both sexes during a large part of their lives, and that both genders display fidelity to the host species where they were born (Camacho *et al.* 2013, Torrico-Bazoberry *et al.* 2014). Since reproductive females of *A. grossa* remain close to and defend their offspring along all their nymphal development, they are exposed to considerable predation risk (Torrico-Bazoberry *et al.* 2014); hence, a defensive role of TAs sequestered from *B. suaveolens* may be suggested. On the other hand, males may also sequester TAs as a defense against predators since males show larger dispersal ranges than females and are thus more exposed to encounters with natural enemies (Bowers 1992, Nishida 2002, Pinto unpublished data).

Given that Sc is the most abundant TA found in different populations of *B. suaveolens* (Freitas *et al.* 1996; Alves *et al.* 2007) and that this compound shows deleterious effects on a variety of phytophagous insects which feed on Solanaceae (Krug & Proksch 1993, Detzel & Wink 1993, Alves 2003, Alcantara 2006, Alves *et al.*

2007, Arab & Trigo 2011, Arab 2012), we propose Sc as the most likely candidate related with plant defense among the TAs identified in *B. suaveolens*. The overall effect of TAs on phytophagous insects may depend on their level of ecological specialization. For example, specialist insects such as adults of the butterfly *Placidula euryanassa* which feed on TA-containing *B. suaveolens* (Solanaceae) contain detectable amounts of TAs, used as chemical protection and host recognition cue. In the case of other insects such *as Miraleria cymothoe* (Nymphalidae), which feeds on *B. suaveolens* without sequestering TAs, the role of compounds such as Sc has been suggested as feeding and oviposition attractants (Nash 1993, Freitas *et al.* 1996, Kitamura *et al.* 2004, Alves *et al.* 2007, Arab 2012).

ASc occurred in the insect in a proportion much higher to that found in the plant and was responsible for the variations between the three body sections. ASc can be produced from Sc in some animals (Werner & Schmidt 1968b, Wada *et al.* 1991), suggesting that a higher concentration of ASc in *A. grossa* could arise from the detoxification of the more toxic Sc (Kitamura 2004). The presence of TAs in the conspicuous pronotum of *A. grossa* supports the idea of allocation of alkaloids in order to deter the attack of predators or to be used as a warning signal of toxicity (Sime 2002, Pinto *et al.* 2011), supporting also the role of the pronotum as a defensive structure in a new chemical context (Wood, 1977, 1993).

Differences in concentrations of TAs between *A. grossa* body sections and *B. suaveolens* leaves, may also be related to differences in the concentration of compounds (e.g. alkaloids) between the phloem sap, where the insect feeds and the leaf tissue which was submitted to analysis (Strong *et al.* 1984). Notwithstanding, at least in the case of hydroxamic acids, a family of secondary metabolites involved in resistance of cereals against phloem-feeding aphids (Niemeyer 2009), concentration of the compounds in the phloem was similar to that in the leaf tissue as a whole (Givovich *et al.* 1994).

We have shown that *A. grossa* sequesters alkaloids from its host-plant and that they are differentially allocated within the body of adult individuals. The ecological roles associated with sequestration of TAs in this species remain unclear; nevertheless, knowing that this membracid prefers to use plants of the Solanaceae and that this family contains a wide variety of alkaloids (McKamey & Deitz 1996, Eckart 2008), this system constitutes an ideal scenario to address questions related to ecological specialization, diversification, sequestration and defensive role of alkaloids, among others.

Nota aclaratoria

El objetivo inicial de este capítulo incluía el estudio de la relación de los metabolitos secundarios, en particular alcaloides, de *B. suaveolens* y *S. ursinum* con los individuos de *A. grossa* desarrollados en ellos. La idea principal era caracterizar el potencial secuestro de alcaloides de forma homologable y comparable entre individuos provenientes de cada planta hospedera. Por ello, se decidió trabajar con cromatografía de gases acoplada a espectrometría de masas, un método sencillo, sensible y versátil ampliamente utilizado en el estudio de diversos tipos de compuestos. Al no obtener resultados en el caso de *Solanum ursinum* (debido probablemente a la baja volatilidad de sus alcaloides), fue imposible efectuar comparaciones cuantitativas entre los insectos desaroolados en ambos hospederos. Se decidión entonces determinar la presencia/ausencia de alcaloides en los insectos, utilizando métodos como cromatografía en capa fina; sin embargo tampoco se obtuvieron resultados con este tipo de protocolo. Al evaluar en este punto otros potenciales métodos, y condiciones para lograr completar el objetivo, las opciones emergentes incluían el uso de cromatografía líquida de alta resolución acoplada a espectrometría de masas, técnica que debía utilizar cantidades de muestras poco realizables para lograr un posible aislamiento e identificación de los alcaloides. Finalmente, una nueva revisión comparativa a nivel literatura demostró una ausencia total de resultados positivos en la comprobación de secuestro de alcaloides por parte de insectos en *Solanum*, probablemente debido a la complejidad molecular de ellos (alcaloides glicosidados esteroidales) y también

dada su baja probabilidad de ser secuestrados por los insectos debido a factores estructurales tales como su alto peso molecular. Se decidió en este punto limitar el trabajo a los alcaloides tropánicos de *B. suaveolens.*

5. CAPÍTULO 4: ESTRUCTURACIÓN GENÉTICO POBLACIONAL EN EL MEMBRACIDO *Alchisme grossa* (HEMIPTERA) EN UN CONTEXTO DE USO DE HOSPEDEROS ALTERNATIVOS

5.1 Resumen

A pesar de que se cuenta con un amplio conjunto de información acerca de patrones de diversificación en una variedad de especies de insectos, aun no se cuenta con información suficiente acerca del funcionamiento de los mecanismos subyacentes a la especiación durante potenciales etapas tempranas de la divergencia. *Alchisme grossa* (Membracidae) es un insecto fitófago que muestra fidelidad hacia sus hospederos de desarrollo, la cual es estrictamente conductual, dado que no existen barreras de otra naturaleza. Los estudios que dan cuenta de esta fidelidad han sido realizados en un setor representativo de los bosques nublados de Bolivia, lugar donde utiliza a *Brugmansia suaveolens* y *Solanum ursinum* – Ambas pertenecientes a la familia Solanaceae - como hospederos alternativos. Para evaluar la potencial existencia de divergencia genómica se utilizaron las enzimas *Pst1* y *Sbf1* para la caracterización de aproximadamente 770 SNPs en 32 muestras. Los resultados mostraron valores de divergencia prácticamente nulos (F_{ST}=0.001), indicando la existencia de una alta similaridad genómica entre los grupos de individuos desarrollados sobre cada especie hospedera. Los resultados son discutidos en términos de los potenciales procesos

explicando la ausencia de estructuración entre las poblaciones de *A. grossa* en cada especie hospedera.

5.2 Introducción

La especialización de insectos fitófagos en el uso de las especies que constituyen sus plantas hospederas es un componente clave durante el proceso de diversificación de especies (Wood 1980, 1993b, Wood & Guttman 1981, 1983, Futuyma & Moreno 1988, Caillaud & Via 2000, Berlocher & Feder 2002, Hernández-Vera *et al.* 2010, Janz *et al.* 2006, Darwell *et al.* 2014); en varios grupos taxonómicos esta especialización muestra una íntima y estrecha relación entre el insecto fitófago y su planta hospedera (Wood & Guttman 1981, Caillaud & Via 2000, Berlocher & Feder 2002, Stireman *et al.* 2005, Hernández-Vera *et al.* 2010, Peccoud *et al.* 2010, Darwell *et al.* 2014). El patrón de uso de las plantas hospederas puede limitar el flujo génico entre poblaciones de insectos que utilizan más de una especie de plantas, lo cual implica algún grado de sesgo en la reproducción asociado al uso de hospederos, por preferencia de apareamiento entre insectos que se desarrollan en la misma especie de planta, o por dificultad en el uso de otras especies de plantas y, con ello, el encuentro de pareja sexual proveniente de tales hospederos, lo cual se suma a la diferenciación fenotípca gatillada por las presiones selectivas impuestas por las propias plantas hospederas, tales como la calidad nutricional de la savia, las defensas químicas de las plantas, y por la presencia de depredadores,

y/o las condiciones microambientales (Singer *et al.* 2004, Dworkin & Jones 2009, Peccoud *et al.* 2010, Darwell *et al.* 2014, Pinto, datos no publicados).

Uno de los requerimientos más importantes para la colonización de un nuevo hospedero involucra que la población colonizadora presente variación genética en rasgos críticos relacionados con el desempeño ("*performance*") que le permitan adaptarse al nuevo hospedero (Futuyma & Moreno 1988, Tilmon *et al.* 1998, Wood *et al.* 1999, Jones 1998, 2004, 2005, Dworkin & Jones 2009). Esta variación genética debería manifestarse en una interacción genotipo x hospedero, donde algunos genotipos se desempeñarán mejor en un hospedero que en otro (Wood *et al.* 1999, Via 1999, Via *et al.* 2000, Berlocher & Feder 2002, Rodríguez & Al-Wathiqui 2011, 2012). En el caso de algunas especies de insectos que usan más de una planta hospedera, se puede observar poblaciones genética y localmente adaptadas a cada especie hospedera (Tilmon *et al.* 1998, Via 1999, Via *et al.* 2000, Stireman *et al.* 2005, Loxdale *et al.* 2011, Darwell *et al.* 2014). Sin embargo, esta estructuración genética puede deberse también a procesos de deriva génica ocurridos después de los eventos fundadores (Downie 1999, Darwell *et al.* 2014) o, probablemente, a efectos de distribución geográfica de las plantas (Hernández-Vera *et al.* 2010).

Los insectos fitófagos que utilizan plantas hospederas alternativas en simpatría, es decir, donde grupos de una misma especie de insectos se encuentran utilizando a la vez más de una especie de planta hospedera dentro de su ambiente (Pinto *et al.*

2015, datos no publicados), pueden estar sometidos a procesos y condiciones que promuevan la especialización ecológica, tales como la sincronización entre las fenologías del insecto y de la planta, la reproducción no aleatoria entre insectos que utilizan cada hospedero y cambios en la duración del ciclo de vida, entre otros (Futuyma & Moreno 1988, Schoonhoven *et al.* 2005, Godoy *et al.* 2006, Torrico-Bazoberry *et al.* 2014). La fidelidad hacia el hospedero de desarrollo o filopatría durante la reproducción y el apareamiento, pueden reducir la migración recíproca en los estadíos iniciales de colonización de nuevos hospederos generando estructuración poblacional asociada a cada hospedero (Wood *et al.* 1999, Via 1999, Stiebens *et al.* 2013).

En el caso de los membrácidos, un grupo de insectos fitófagos, algunos individuos, como los del complejo de *Enchenopa binotata*, presentan un alto grado de fidelidad al hospedero, con dispersión limitada y una marcada tendencia hacia aparearse y ovipositar en su hospedero natal (Wood 1980). Estos dos rasgos de historia de vida podrían reducir el flujo génico después de que un cambio de hospedero haya ocurrido (Sattman & Cocroft 2003); sin embargo, algún apareamiento e intercambio son aún posibles debido al solapamiento parcial de los periodos de reproducción y a la dispersión ocasional de individuos, principalmente de machos en busca de apareamiento (Sattman & Cocroft 2003).

Alchisme grossa (Hemiptera: Membracidae) es un membrácido perteneciente a la tribu Hoplophorionini que se caracteriza por presentar cuidado maternal extendido y una alta preferencia hacia las plantas de la familia Solanaceae (McKamey & Deitz 1996, Camacho *et al.* 2014, Torrico-Bazoberry *et al.* 2014). En los bosques nublados de la región de Incachaca en Bolivia, este insecto se encuentra utilizando dos especies de plantas hospederas que subsisten en simpatría: *Brugmansia suaveolens* y *Solanum ursinum* (ambas de la familia Solanaceae) (Torrico-Bazoberry *et al.* 2014). Estudios previos indican que los individuos de esta especie presentan fidelidad alimenticia hacia su planta hospedera de origen, y además presentan baja tasa de dispersión entre plantas de las dos especies (Pinto et al., datos no publicados); sin embargo, se desconoce si tales patrones conductuales observados también se manifiestan en la estructuración genético poblacional de esta especie asociada al uso de hospederos distintos. En este contexto, el objetivo de este trabajo fue analizar la diversidad genética de individuos de *A. grossa* en cada hospedero alternativo, determinando si existe estructuración poblacional en los grupos asociados a cada planta.

5.3 Materiales y Métodos

Colecta de muestras y extracción de ADN

Para el muestreo se utilizaron adultos tenerales de una población de *A. grossa* de la localidad de Incachaca en Cochabamba, Bolivia (17 °13' S to 65°49' W; 2.450 msnm) ubicada en la región biogeográfica de los Yungas. Se colectaron 32 individuos, 16 desarrollados en la planta hospedera *B. suaveolens* y los otros 16 en *S. ursinum*. Los individuos fueron sacrificados por congelamiento en un refrigerador a -4°C y preservados en etanol al 96 % a -20°C para posteriores análisis moleculares.

Preparación de librerías y secuenciación

Previamente a la extracción de ADN, el pronoto, alas, patas y abdomen de cada individuo fueron removidos utilizando un bisturí. El ADN del torax completo de cada individuo fue extraído utilizando el Kit de Extracción para Sangre y Tejidos Qiagen DNAeasy. Dos librerías de *Restriction-site associated DNA sequencing* (RADs) fueron preparadas siguiendo el protocolo de Etter *et al.* (2011), usando entre 500 y 1000 ng de DNA por muestra y dos enzimas de restricción distintas, *Sbf*1 y *Pst*1 respectivamente. Las librerías de RADs fueron preparadas con una combinacion de dos tipos de *barcodes*: 1) un *barcode* P1 formado por un total de 8pb para un total de 16 *barcodes* diferentes y 2) un *barcode* P2 formado por 8 índices distintos. El DNA fue fragmentado utilizando un sonicador Covaris con un ciclo de trabajo al

10%, una intensidad de 4 y 200 ciclos por evento durante 48 s. Las librerías fragmentadas fueron corridas en un gel de agarosa, donde se seleccionaron bandas en un rango entre 300 y 500 bp. Dicho producto fue enriquecido con 16 ciclos de amplificación en 10 reacciones de PCR, las cuales contenían 5 µl de Master Mix de alta fidelidad de PCR con un buffer HF, 0.2 µl de cada primer amplificado (10 µM), 2µL de la librería (9ng/ µL) y 2.6 µL de agua (10 µL en total). Las dos librerías fueron secuenciadas en una sola línea de secuencia (64 individuos: 32 con *Pst1* y 32 con *Sbf1*) en una Illumina HiSeq2000 de acuerdo a las instrucciones del fabricante.

Filtrado y control de calidad de las lecturas de secuencia

EL filtrado de secuencias fue realizado utilizando el comando *process_radtags* en el programa Stacks 1.08 (Catchen *et al.* 2011, 2013). Este comando elimina: 1) lecturas de baja calidad (valor de *phred* <10 dentro de una ventana de 15 pb), 2) lecturas con índices ambiguos en las primeras 8 pb, 3) lecturas ambiguas entre 9 y 15 pb correspondientes en secuencias del sitio de corte de las enzimas de restricción usadas (*Pst1* y *Sbf1*) y 4) lecturas que incluyan secuencias de los adaptadores. Adicionalmente, el comando *clone-filter* en en el programa Stacks 1.08 fue utilizado para filtrar pares de lecturas que son exactamente iguales, dado que se esperaba que estas son producidas por copias del mismo fragmento durante las amplificaciones de PCR.

Ensamblaje

En ausencia de un genoma de referencia para *A. grossa*, las lecturas fueron ensambladas *de novo* usando el comando *denovo_map.pl* en Stacks 1.08. Para el análisis global, el número de lecturas en bruto requeridas para formar un Stack o Locus (m) fue establecido en 3 y el número de nucleótidos discordantes permitidos entre dos Stacks o Locus (M) fue establecido en 2.

Análisis genética de poblaciones

Por consistencia, niveles similares de filtrado fueron aplicados para todos los análisis. Las muestras fueron inicialmente agrupadas por planta hospedera (BS o SU) en stacks o loci con cobertura (*coverage*) ≥ 10x en ≥ 12 individuos por planta hospedera. La herramienta *population* en el programa Stacks 1.08 fue usada para calcular *Fst*, que mide la intensidad de flujo genético entre pares de poblaciones. Los resultados de *Fst* fueron graficados usando *scripts* propios en paquete de análisis de datos R (R Development Core Team 2008).

Análisis de clustering (agrupación)

Los análisis de agrupamiento fueron realizados para explorar en detalle la existencia de estructura poblacional. Todos los loci con *coverage* ≥ 10x en ≥ 12 individuos fueron considerados para este análisis; sin embargo, solo se tuvo en cuenta SNPs independientes (el primer SNP de cada loci). El programa STRUCTURE versión 2.3.4 (Pritchard *et al.* 2000) fue utilizado para estos análisis,

considerando un modelo mixto (*admixture*) con frecuencias correlacionadas (Falush *et al.* 2003). Dicho análisis se le aplicó un periodo de quemado de 100.000 iteraciones MCMC, seguidas de 100.000 iteraciones para distintos valores de K (posible numero de grupos genotípicos) entre 1 a 5 y 10 réplicas por análisis . El mejor valor estimado *K* fue seleccionado a partir de dos criterios distintos: 1) el número de agrupamientos genéticos que corresponde al valor de K con el mayor ln Pr (X/K) (Pritchard *et al.* 2000) y 2) se usó el estadístico *ad hoc* ΔK (Evanno *et al.* 2005), teniendo en cuenta que el mismo no se aplica cuando K=1.

5.4 Resultados

Filtrado y ensamblado de secuencias

En el caso del uso de la enzima *Pst1*, se obtuvo un total de 11,437,562 lecturas de 200 pares de bases. De estas, un 3,47 % fueron descartadas debido a su baja calidad , 5% fueron descartadas por tratarse de clones PCR, 0.41 % debido a que eran sitios de restricción ambiguos y 0.82 % debido a indices ambiguos. Globalmente, se retuvieron 11,167,379 secuencias (98 % de las secuencias en bruto).

En el caso del uso de la enzima *Sbf1*, se obtuvo un total de 7,917,506 lecturas de 200 pares de bases. De estas, un 3.44 % fueron desacartadas debido a su baja calidad , 5% fueron descartadas al tratarse de clones PCR, 1.44 % debido a que eran

sitios de restricción ambiguos y 0.92 % debido a índices ambiguos. Globalmente, sde retuvieron 7,686,246 secuencias (97 % de las secuencias en bruto).

Con el uso de esta enzima se lograron identificar un total de 278 SNP, con un total de 108 loci, y un *coverage* promedio de 20x por locus. El uso de la enzima *sbf1* permitió identificar un total del 492 SNP con 137 loci en total. con un coverage promedio de 20x por locus. En total entre las dos enzimas de restricción se logró identificar 770 SNP en 245 loci.

Estadística de genética de poblaciones

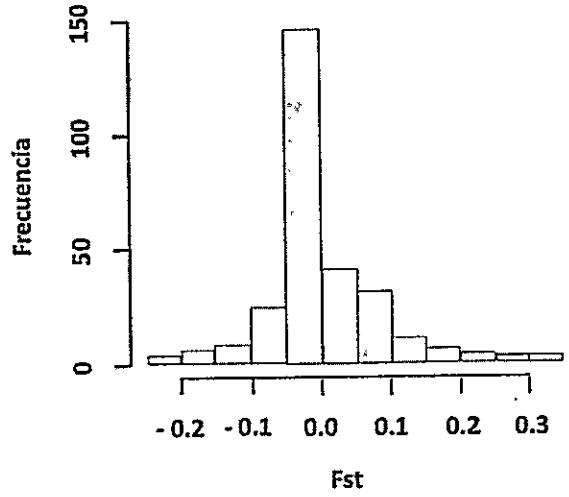
El número de sitios, número de sitios variantes, número y proporción de sitios polimórficos, número de individuos muestreados, diversidad nucleotídica y heterozigosidad esperada en las dos poblaciones consideradas en este estudio, *B. suaveolens* y *S. ursinum*, utilizando las enzimas de restricción *Pst1* y *Sbf* 1 son reportados en la Tabla 10.

Tabla 10. Número de sitios, número de sitios variantes, número y proporción de sitios polimórficos, número de individuos muestreados, diversidad nucleotídica y heterozigosidad esperada en las dos poblaciones consideradas en este studio Pop 1 (*B.* suaveolens) Pop 2 (S. ursinum), utilizando las enzimas de restricción *Pst1* y *Sbf1*.

	Pst1 (N=32)		*Sbf1* (N=32)	
	Pop 1	**Pop 2**	**Pop 1**	**Pop2**
Número de sitios	19447	19447	26740	26740
Número de sitios variantes	279	270	495	493
Número de sitios polimórficos	215	214	383	318
% de sitios polimórficos	1,1	1,1	1,4	1,1
Individuos muestreados	14,6	14,1	12,4	12,6
Diversidad nucleotídica	0,003	0,003	0,003	0,003
Heterozigosidad esperada	0,002	0,002	0,003	0,003

El F_{ST} global fue calculado en 0.00100516. La distribución de valores de F_{ST} por SNP estuvo caracterizada por una moda alrededor de 0 en ambas enzimas y una cola extendiéndose hasta 0.4 (Fig. 6). En el caso de *Sbf1*, 11 loci (outliers) fueron significativamente distintos entre las dos poblaciones (< 0.05), lo cual indica que pueden tomarse en cuenta como candidatos para participar en el aislamiento genético en *A. grossa*.

a)



b)

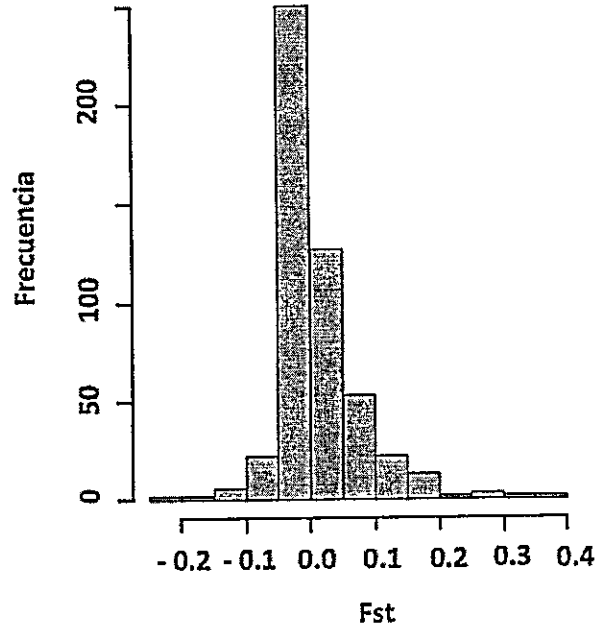


Figura 6. Distribución global de los Fst estimados a) utilizando la enzima de restricción *Pst1* y b) Utilizando la enzima de restricción *Sbf1*.

Análisis de agrupamiento

Considerando un solo SNP por *stack* o locus, un total de 112 loci para *Pst1* y 162 para *Sbf1* fueron retenidos. Para distintos valores de K no se encontraron clusters genéticos asociados con el agrupamiento fenotípico (en qué planta se alimenta *A. grossa*) para ninguna de las dos enzimas (Fig. 7).

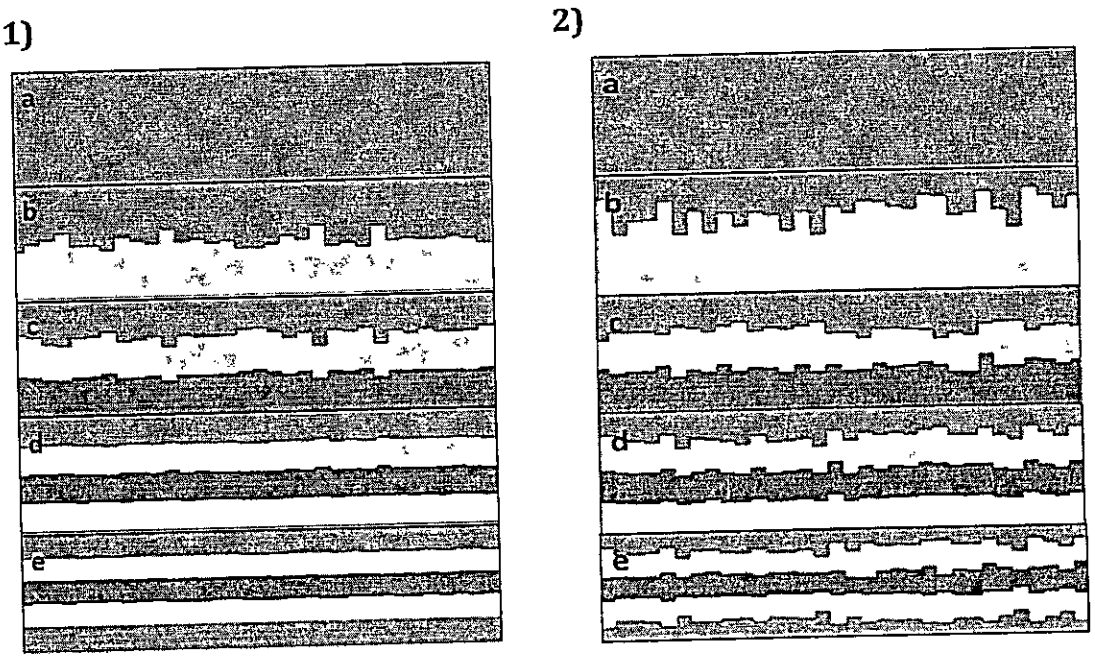


Figura 7. Resultados del análisis se agrupamiento para todo el set de datos asumiendo distintos valores de K (a=1, b=2 c=3, d=4 y c= 5) a) Resultados para la enzima *Pst1* y b) resultados para la enzima *Sbf1*.

5.5 Discusión

No se encontró ningún patrón de agrupamiento o estructuración genética en los individuos de *A. grossa* subsistiendo en ambas plantas hospederas, donde la distribución de los valores de F_{ST} estimados a partir de los SNP presentó una moda alrededor de 0 con aproximadamente el 99 % de las estimaciones. Sin embargo, se identificaron 11 loci que constituyen outliers de F_{ST}, los cuales podrían constituirse en candidatos relacionados con la adaptación diferencial de *A. grossa* sobre cada especie de planta hospedera.

El uso de plantas hospederas alternativas en simpatría en insectos fitófagos requiere inicialmente de la existencia de variación genética en rasgos relacionados a la adecuación biológica (Tilmon *et al.* 1998, Dworkin & Jones 2009), lo que, después de un proceso de colonización exitosa de un nuevo hospedero, podría generar algún nivel de estructuración poblacional de los insectos asociados a cada especie de planta (Guttman *et al.* 1981, Guttman *et al.* 1989, Funk *et al.* 2002, Stireman *et al.* 2005). En el caso de *A. grossa*, no se observó una estructuración poblacional asociada al uso de dos plantas hospederas alternativas simpátricas, a pesar de que estudios previos mostraron que los individuos desarrollados en cada hospedero presentaron una marcada preferencia para alimentarse, dispersarse y ovipositar en plantas de la especie donde se desarrollaron (Pinto, datos no publicados).

Esta falta de estructuración genético poblacional podría deberse a varios factores. Por ejemplo, podría ser explicada por la hipótesis planteada por Wood (1980) que sugiere que mientras más distante es la relación filogenética entre las plantas hospederas colonizadas por insectos fitófagos, mayor sería la presión de selección para desarrollar pozas génicas distintas compatibles con cada hospedero en los insectos, es decir, el generar poblaciones de insectos en cada hospedero con una información genética particular. Las plantas hospederas de *A. grossa, B. suaveolens* y *S. ursinum,* son plantas emparentadas pertenecientes a la misma familia taxonómica (Solanaceae), por lo que es probable que la presión de selección ejercida por las plantas hospederas no sea lo suficientemente fuerte para provocar la selección de pozas génicas específicas a cada hospedero. Sin embargo, cada especie hospedera tiene sus propias características nutricionales, químicas (ej. defensas) y microambientales particulares ante las cuales se encuentran expuestos los individuos de *A. grossa* (Pinto, datos no publicados), por lo que sería esperable encontrar al menos una estructuración genética inicial entre hospederos. Los alelos potencialmente bajo selección encontrados en los insectos desarrollados en cada plantas hospedera podrían estar sujetos a presiones de selección diferenciales dependiendo del hospedero donde se desarrolla *A. grossa*.

Teniendo en cuenta lo anteriormente expuesto, es probable que la falta de estructuración poblacional entre individuos desarrollados en ambas plantas hospederas nos permita hipotetizar o sugerir que el evento de colonización de *B.*

suaveolens por individuos de *A. grossa* se constituya en un evento relativamente reciente, de modo que el tiempo de colonización no ha sido el suficiente para que alelos comunes entre grupos de insectos desarrollados en cada planta desaparezcan y las poblaciones se empiecen a diferenciar genéticamente como fruto del uso de ambientes distintos a través de diversos procesos (e.g. aislamiento reproductivo, selección natural). Si se considera la falta de diferencias encontradas entre plantas hospederas en el desempeño y la respuesta demográfica, y en los patrones de selección natural en *A. grossa* (Pinto, datos no publicados; Torrico-Bazoberry, datos no publicados), además de observaciones adicionales de *S. ursinum* como hospedero expclusivo en otras regiones de Bolivia, el escenario de colonización reciente de *B. suaveolens* sugerido parece verse aún más reforzado.

Por otro lado, se ha planteado que la eclosión de los huevos de los membrácidos está correlacionada con la fenología del hospedero (Wood 1980, Wood 1993a, Lin 2006, Godoy *et al.* 2006), lo que deriva en que las poblaciones de insectos en una especie de planta podrían desfasarse respecto a las poblaciones que se encuentran sobre otro hospedero (Wood 1993a, 1993b). Más aún, el crecimiento y desarrollo de las ninfas podría variar entre especies de hospederos, dependiendo de la calidad de nutrientes de la planta (Wood 1980) o de las condiciones ecológicas microambientales que la planta otorga (Pinto *et al.* 2015, en preparación). Estas diferencias en el tiempo de maduración pueden magnificar el efecto de la fenología en la eclosión de huevos hasta el punto donde la mayoría de los insectos de un

hospedero ya se han apareado antes de que los del otro hospedero se hayan vuelto sexualmente receptivos (Wood 1980, 1993b), lo que se vería reflejado en la estructuración genética resultante entre individuos de cada planta hospedera. En el caso de *A. grossa*, la eclosión de los huevos no parece estar correlacionada con la fenología de la planta hospedera; sin embargo, el tiempo de crecimiento y desarrollo de las ninfas sí varía entre hospederos (Torrico-Bazoberry *et al.* 2014, Pinto, datos no publicados). Sin embargo, parece ser que la diferencia en el tiempo de desarrollo de los individuos de *A. grossa* entre plantas hospederas no tendría un efecto en la reducción del flujo genético entre individuos desarrollados sobre una u otra planta, posiblemente porque el periodo de receptividad de las hembras de *A. grossa* desarrolladas en cada planta podría variar mucho, o porque la longevidad de los machos también varía y no afecta la reproducción entre individuos de ambas plantas, además de la opción de que las cohortes de aproximadamente la misma edad y/o estado reproductivo, sean sincrónicas en ambos hospederos en condiciones naturales. Para determinar los efectos de la diferencia del tiempo en el desarrollo de *A. grossa* en la reproducción entre individuos de ambas plantas hospederas, es necesario desarrollar estudios detallados sobre la conducta y fenología reproductiva de esta especie.

Existen mecanismos conductuales de evitación del interapareamiento de membrácidos que viven en distintas especies hospederas , como aquellos descritos por Sattman & Cocroft (2003) para *E. binotata*, en que los machos sesgan su

conducta de búsqueda de pareja dependiendo si se encuentra sobre su planta hospedera o sobre una planta no-hospedera; en esta especie, cuando los machos producen señales vibracionales sobre una especie no-hospedera, emiten menos señales por evento, y se demoran más tiempo en empezar a producir las señales que cuando se encuentran sobre una especie de planta hospedera. Estas conductas podrían reducir la probabilidad de apareamiento de un macho con hembras de otro hospedero. En este contexto, estudios que se enfoquen en la conducta reproductiva de los machos de *A. grossa* resultarían importantes para indagar desde otro enfoque una explicación mecanicista a la ausencia de estructuración genético poblacional.

6. DISCUSIÓN GENERAL

Dispersión - Fidelidad

La fidelidad alimenticia en *A. grossa* muestra resultados relativamente complejos de interpretar desde el punto de vista de la preferencia y aceptación. Los datos de preferencia agrupados por sexo y sin agrupar, indican una tendencia general a preferir *B. suaveolens*; sin embargo, al analizar cada tratamiento por separado (1) BF: hembras de AG desarrolladas BS, 2) BM: machos de AG desarrollados en BS, 3) SF: hembras de AG desarrolladas en SU y 4) SM: machos de AG desarrollados en SU), sí se detecta preferencia en todos ellos hacia el hospedero de desarrollo. Ambos hechos posiblemente estén relacionados con la fuerte preferencia mostrada por parte de los membrácidos desarrollados *sobre B. suaveolens* comparada con una preferencia más débil de aquellos desarrollados sobre *S. ursinum* (Fordyce *et al.* 2011). Sin embargo, al analizar individualmente el patrón de preferencia en cada tratamiento o en cada especie hospedera, se evidencia la existencia de fidelidad hacia la planta de desarrollo de los individuos, aunque se debe tomar en cuenta que estos dos últimos esquemas de análisis tienen los Criterios de desviación de Información (DIC) más altos, lo cual los convierte en los menos robustos a nivel análisis. Por otro lado, las pruebas de aceptación muestran que no existirían barreras fisiológicas para el uso de cualquiera de los dos hospederos, hecho que se encuentra apoyado por un desempeño similar de *A. grossa* en ambos hospederos

como resultado de un estudio que involucró trasplantes recíprocos (Pinto, datos sin publicar).

La antigüedad de la relación de *A. grossa* con cada uno de sus hospederos puede en parte explicar el patrón de uso observado en este trabajo; dado que la Yunga está caracterizada por contener una alta riqueza de solanáceas y que este membrácido ha sido reportado en 5 puntos del país utilizando solo *S. ursinum,* se sugiere que el uso de este hospedero sea de mayor antiguedad comparado con *B. suaveolens* (Courtney *et al.* 1989, Singer 2002, McKamey & Deitz 1996, Camacho *et al.* 2014, Martins & Barkman 2005, Pinto, datos sin publicar). Sin embargo, también se debe tomar en cuenta las importantes diferencias entre los hospederos en cuanto a ambientes de desarrollo (e.g. diferencias químicas, morfológicas, ecológicas) las cuales también puede jugar un rol promoviendo el desarrollo de fidelidad hacia cada una de las plantas utilizadas como hospederas (Bernays & Chapman, 1994, Wei *et al.* 2000, Schoonhoven *et al.* 2005).

Por otro lado, los resultados obtenidos a través del marcaje y recaptura mostraron valores similares en el éxito en recapturas obtenidos en estudios similares con otros membrácidos (Wood & Dowell 1985), donde la proporción de sexos también mostró estar ligeramente sesgada hacia las hembras. Las grupos de individuos desarrollados sobre cada hospedero al parecer forman poblaciones relativamente agregadas y altamente fieles a la especie de planta de desarrollo. En todos los casos

las hembras fueron más móviles que los machos, factor que podría ayudar a evitar escenarios de endogamia en las poblaciones locales (Wood & Dowell 1985, Funderburk & Mack 1989). Sobre la base de estos resultados se propone que la fidelidad en *A. grossa* hacia su hospedero sería en gran parte explicada por rasgos conductuales, dada la ausencia de barreras (e.g. geográficas, mecánicas, químicas) que les impidan utilizar uno u otro hospedero.

Demografía y cuidado parental

Las diferencias en la duración del ciclo de vida de *A. grossa* en cada uno de los dos hospederos estudiados puede ser un producto directo del patrón de fidelidad de uso de los mismos, involucrando además potenciales efectos en la adecuación biológica, ecología y biología de los individuos desarrollándose en cada planta hospedera (Wood 1993a, Futuyma & Moreno 1988, Jaenike 1990). Estas diferencias, sumadas a la sincronía de las cohortes reportada para *A. grossa*, podría promover un futuro escenario de aislamiento reproductivo de grupos viviendo sobre cada especie de planta hospedera y, de ser así, también se esperaría su diversificación via especiación ecológica tal como ha sido propuesto para otros membrácidos (Futuyma & Moreno 1988, Wood *et al.* 1999, Lin & Wood 2002, Cocroft *et al.* 2008, 2010).

La calidad del hospedero como ambiente de desarrollo afecta directamente la dinámica poblacional de los insectos que viven en ellos, proceso que ha sido poco

abordado en condiciones naturales (Larsson *et al.* 2000, Awmack & Leather 2002, Schoonhoven 2005, García-Robledo & Horvitz 2011). Esta investigación exploró atributos demográficos en *A. grossa* asociados al uso de hospederos alternativos evidenciando que el desempeño y la dinámica de los grupos de insectos viviendo sobre cada planta hospedera no se diferencia, a la vez que la estasis (permanencia) se constituyó en el parámetro con mayor aporte a cambios en la tasa de crecimiento poblacional de los grupos estudiados. Tal patrón ha sido observado con las mismas tendencias en diversos grupos animales y vegetales (Caswell 2001, Enright *et al.* 1995, Pfister 1998). En este sentido, queda en evidencia el hecho de que los genotipos de *A. grossa* subsistiendo en cada hospedero pueden compartir algún factor ambiental que explique parcialmente la relación costo/beneficio de estar en uno u otro hospedero, con la posibilidad de mantener poblaciones viables en cada uno. En este sentido, el factor común más relevante estaría relacionado con el cuidado maternal ejercido por las hembras de *A. grossa* a lo largo del desarrollo ninfal.

El cuidado parental está ampliamente distribuido en 13 órdenes, y en al menos 47 familias de insectos (Choe & Crespi 1997, Lin 2006, Mass & Kolliker 2008, Trumbo 2012). En los membrácidos, la forma de cuidado parental más común consiste en la permanencia y vigilancia de la madre cerca de la progenie, y su protección contra los depredadores y parasitoides (Lin *et al.* 2004, Godoy *et al.* 2006, Lin 2006). El cuidado maternal en estos insectos muchas veces tiene corta duración y la tarea es

delegada a insectos mutualistas himenópteros (principalmente hormigas) que se ocupan del cuidado y defensa de la progenie, recibiendo a cambio una sustancia azucarada de las ninfas y/o adultos (Bristow 1983, Wood 1984, Moreira & Del-Claro 2005). Sin embargo, membrácidos de la tribu Hoplophorionini – donde se encuentra *A. grossa* - no interactúan con hormigas, debido posiblemente a que se distribuyen mayormente en las zonas montanas y submontanas húmedas de los Andes tropicales (Mckamey & Deitz 1996, Lin 2006), donde la presencia de hormigas es muy baja (Janzen *et al.* 1976, Olmstead & Wood 1990).

En esta tribu, el cuidado de las ninfas depende exclusivamente de las madres y se extiende hasta que la progenie alcanza el estadío adulto (Mckamey & Deitz 1996, Lin *et al.* 2004). Las especies de esta tribu exhiben el mayor grado de inversión maternal entre los membrácidos (Mckamey & Deitz 1996) y por tanto, en ellas han evolucionado adaptaciones conductuales (Wood 1974, Cocroft 2002, Lin 2006), comunicacionales (Nault *et al.* 1974, Cocroft 1999a, Ramaswamy & Cocroft 2009) y morfológicas (Funkhouser 1917, Creao-Duarte & Sakakibara 1997, Lin 2006, Camacho *et al.* 2013) que les permiten defender efectivamente a su progenie. Los antecedentes que respaldan la compleja evolución del cuidado maternal en Hoplophorionini apoyan los hallazgos de este trabajo; dada la vital importancia del cuidado maternal para la sobrevivencia de la progenie en *A. grossa,* las diferencias en la calidad del hábitat asociada a cada hospedero pueden verse balanceadas por

el cuidado maternal, similar cualitativa y cuantitativamente en ambas especies de plantas hospederas.

Química

La presencia de tres tipos de alcaloides derivados del tropano (TAs) (3-fenilacetoxi-6,7-epoxitropano, apoescopolamina y escopolamina) fueron reportadas para hembras y machos de *A. grossa* - en similar concentración en ambos sexos - desarrollados en *B. suaveolens,* hecho que sugiere estar ligado a un secuestro pasivo por parte de los insectos (Camacho *et al.* 2014, Alves *et al.* 2007). Dado que este membrácido muestra fidelidad al hospedero de desarrollo a lo largo de su vida, el patrón de secuestro observado podría también estar relacionado con la química propia de cada una de la plantas hospederas. Los roles de los TAs secuestrados en la biología y ecología del insecto, se han relacionado con diversos procesos ecológicos; por ejemplo, la escopolamina - alcaloide más abundante en *B. suaveolens* – es un compuesto deletéreo contra insectos fitófagos generalistas, pero en casos donde es secuestrado por insectos más especializados puede transformarse en señal de reconocimiento que les permite identificar el hospedero para oviposición y/o alimentación, o como defensa antidepredatoria (Bowers 1992, Nishida 2002, Kitamura *et al.* 2004, Alves *et al.* 2007, Arab & Trigo 2011, Arab *et al.* 2012).

La alta cantidad de escopolamina secuestrada en la zona del pronoto de *A. grossa* es un indicador de que esta estructura puede estar cumpliendo efectivamente roles defensivos mas allá de su dureza o forma (Wood, 1977, 1993a). Por otro lado, dado que muchos membrácidos de esta tribu están asociados con solanáceas, la presencia de alcaloides y su distribución en los insectos puede contribuir a dilucidar el fenómeno de la especialización ecológica y diversificación en el grupo mediante su uso como rasgo para la reconstrucción histórica de estas interacciones (e.g. filogenias, método comparado) (McKamey & Deitz 1996, Eckart 2008).

Genética

Aunque la evidencia encontrada muestra diferencias conductuales asociadas al desarrollo de poblaciones independientes sobre cada planta hospedera (e.g. fidelidad alimenticia, dispersión casi exclusiva sobre hospederos de desarrollo), al hacer un acercamiento al aspecto genético se evidencia ausencia de estructuración poblacional en *A. grossa.* Este aspecto se discute en términos relacionados al efecto de la cercanía filogenética de los hospederos, o el potencial corto tiempo transcurrido desde la colonización de alguno de los hospederos (potencialmente *B. suaveolens*) (Wood 1980, Stireman *et al.* 2005). Al mismo tiempo se presume un proceso de estructuración poblacional actualmente en curso, dado que los individuos ya muestran barreras conductuales que pueden evitar el cruzamiento entre insectos desarrollados sobre una u otra planta y, a la vez, el tiempo de desarrollo de los individuos que varía entre hospederos, hecho que puede

promover la generación de poblaciones aisladas reproductivamente en simpatría (Torrico – Bazoberry *et al.* 2014, Pinto datos no publicados).

7. CONCLUSIONES

A continuación se presentan las conclusiones referidas a cada objetivo específico de estudio:

1) Caracterizar el patrón de dispersión de individuos de *A. grossa* a niveles intra e interhospedero, utilizando adultos desarrollados sobre ambas plantas, y evaluar el nivel de fidelidad alimenticia por parte de *A. grossa* hacia sus hospederos de desarrollo.

Los patrones de dispersión de *A. grossa* y su conducta alimenticia demostraron que este insecto ha desarrollado fidelidad hacia la especie de planta hospedera donde vive, sin que esto implique la existencia de barreras que impidan que los individuos se puedan alimentar de cualquiera de los dos hospederos alternativos en los que es observado en la localidad de estudio.

2) Explorar la tasa de crecimiento poblacional asociada a los individuos de *A. grossa* desarrollados sobre uno u otro hospedero, con el fin de indagar la calidad del ambiente que constituye cada planta hospedera para la adecuación biológica de los individuos a nivel poblacional.

Aunque cada una de las especies de planta hospedera constituye un ambiente ecológicamente distinto para el desarrollo de *A. grossa*, esto no se refleja en diferencias en el desempeño ni en la demografía de las poblaciones de *A. grossa* desarrolladas sobre cada una de ellas. Por otro lado, el cuidado maternal demostró ser desplegado con la misma calidad/intensidad en ambos hospederos, comportamiento que representaría un factor de equilibrio de la relación costos/beneficios de vivir asociados a cada especie de plantas.

3) Caracterizar la relación entre la química de las plantas hospederas y la de los individuos de *A. grossa* desarrollados en cada una de ellas.

Se comprobó, el secuestro de tres tipos de alcaloides por parte de machos y hembras de *A. grossa* desarrolladas en *Brugmansia suaveolens*. Dicho proceso sugiere estar relacionado con distintos aspectos de la ecología y biología de esta especie como la defensa antidepredadora y la preferencia de hospedero.

4) Determinar el nivel de estructuración genético - poblacional en *A. grossa* asociado al uso de ambos hospederos, para evaluar la existencia de diferenciación genética y la magnitud de flujo genético entre individuos desarrollados en cada uno de los hospederos.

Se determinó una ausencia de estructuración genético - poblacional entre grupos de individuos desarrollados en cada una de las plantas hospederas, factor que en conjunto con el resto de los resultados previamente obtenidos, permiten preguntarnos si la interacción de *A. grossa* con sus plantas hospederas, representa la etapa inicial de un proceso de especialización ecológica. Por lo mismo, esta especie de insecto constituye un interesante modelo para el estudio de la evolución de la especialización ecológica en la interacción insecto - planta.

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