Semioquímicos como mecanismo de aislamiento reproductivo precopulatorio entre las especies hermanas *Dendroctonus frontalis* y *Dendroctonus mesoamericanus* (Curculionidae: Scolytinae).

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RESUMEN

Dendroctonus frontalis Zimmermann y *D. mesoamericanus* Armendariz-Toledano & Sullivan son especies primarias cercanamente relacionadas que comúnmente coexisten en el mismo hospedero, lo cual es poco evidente en la naturaleza y poco entendida. Se exploró la influencia de los semioquímicos como posibles mecanismos de aislamiento reproductivo que operan entre estas dos especies. Con el uso de estímulos naturales y sintéticos relacionados a las hembras se realizaron en una primera etapa, bioensayos con machos y pruebas electrofisiológicas, con lo que se obtuvo el perfil de semioquímicos de las hembras de cada especie. En una segunda etapa se realizaron pruebas en campo de atracción cruzada. Los resultados indican que los machos de cada especie discriminan entre las hembras con- y heteroespecíficas a través de reconocer los semioquímicos relacionados a estas. Al parecer la *endo*-brevicomina y el ipsdienol producidos por las hembras de *D. mesoamericanus* contribuyen de manera importante en la mediación de la discriminación. Los resultados sugieren que entre los machos de cada especie presentan estrategias diferentes para el reconocimiento de la pareja. *D. frontalis* después de responder a los semioquímicos relacionados al sitio de agregación y de su arribo sobre el hospedero realiza el reconocimiento formal de la pareja, mientras que *D. mesoamericanus* lo hace con mayor grado de especificidad antes y después del arribo al hospedero. Los resultados obtenidos permiten concluir que los semioquímicos funcionan como un mecanismo de aislamiento reproductivo precopulatorio, no obstante este mecanismo no es absoluto.

Palabras clave: mecanismos de aislamiento reproductivo, especies hermanas, *D. frontalis*, alocronía de ataque, feromonas.

CAPÍTULO I

Introducción general

El género *Dendroctonus* Erichson (Coleoptera: Curculionidae: Scolytinae) es un grupo de descortezadores integrado por 20 especies, 18 de las cuales se localizan en Norte y Centroamérica, y dos en Europa y Asia (Wood, 1982; Armendáriz-Toledano et al., 2015). Su distribución geográfica se explica en gran parte por la especificidad y abundancia sobre las especies de Pinaceas que explotan (*Larix* Mill, *Picea* Link, *Pinus* L. y *Pseudotsuga* Carriére) (Kelley y Farrell, 1998) así como también a su distribución geográfica (Zúñiga et al., 1999; Salinas-Moreno et al., 2004).

Los descortezadores de este género representan un factor importante de disturbio para los bosques de coníferas, debido a que culminan con el proceso de deterioro de su salud al detectar y matar árboles susceptibles para su colonización (Wood, 1982; Coulson y Klepzig, 2011). La susceptibilidad de las comunidades de pinos está relacionada con el alto grado de estrés al que están sujetos debido a factores ambientales (p. ej. la exposición a sequías prolongadas, incendios, tormentas eléctricas y cambio climático) (McNulty y Aber, 2001, Bentz et al., 2010), a la estructura de la comunidad de pinos, principalmente los relacionados a la densidad (Fiedler y McKinney, 2004) y a factores antropogénicos (p.ej. la extracción selectiva de especies para la obtención de productos maderables y no maderables, la tala ilegal, el cambio de uso de suelo) (Savage, 1994). De tal manera que estos insectos pueden también ser vistos como indicadores del deterioro de salud forestal (Fetting, 2012).

En condiciones de baja densidad poblacional (población endémica) donde se considera normal y parte del ecosistema, los descortezadores promueven la salud forestal dentro de

una comunidad de pinos, ya que eliminan principalmente aquellos individuos sobre maduros o enfermos. Sin embargo, un incremento de pinos susceptibles ocasionado por algún tipo de estrés puede promover su explotación poblacional (población epidémica) (Six y Bracewell, 2015). En estas condiciones el estudio de los descortezadores toma relevancia debido a que sus efectos han ocasionado la pérdida de grandes extensiones de bosque de pinos en un sólo evento de infestación (Billings et al., 2004). En México, por ejemplo, el daño se extendió en el 2004 a 12,670 hectáreas dañadas por descortezadores (CONAFOR, 2007), lo que representa un factor importante en la fragmentación y estabilidad ecológica de los bosques de pino.

1. Comunicación química para la obtención del recurso en el género Dendroctonus

En insectos descortezadores del género *Dendroctonus* se ha reconocido la comunicación visual, táctil, gustativa y sónica, que es empleada en alguna etapa de la colonización y establecimiento de la progenie (Byers y Zhang, 2011). Sin embargo la comunicación sensorial basada en la olfacción es empleada en prácticamente todo el proceso de colonización del hospedero y representa quizás el sistema sensorial más desarrollado y estudiado en estos insectos. En la obtención y repartición del recurso es importante la producción de feromonas, por parte de las especies de descortezador, y el reconocimiento de kairomonas liberadas por el hospedero (Byers y Zhang, 2011).

Las hembras del género *Dendroctonus* son las que a través del vuelo localizan y exploran nuevos hospederos (Gara, 1967; Gara y Coster, 1968). Las teorías sobre la elección del hospedero indican que las hembras reconocen volátiles (terpenos principalmente) que el hospedero libera, lo cual es probablemente un indicador de alguna condición de enfermedad o que su sistema de defensa se encuentra comprometido (Byers,

1989). Una segunda teoría establece que las hembras deben elegir al hospedero a prueba y error, lo que implica el aterrizaje sobre cualquier hospedero, palpar su corteza, y probar la condición de su defensa toxica y mécanica, es decir, la fuerza de expulsión de la resina (Byers, 1999). Probablemente ambas teorías ocurran de manera complementaria (Byers y Zhang, 2011).

Las hembras pioneras una vez que han logrado establecerse en el interior del hospedero producen y liberan feromonas que promueven un rápido comportamiento gregario de conespecíficos para superar la defensa tóxica y mecánica del hospedero (Six y Bracewell, 2015). Las feromonas liberadas por las hembras también promueven la atracción de los machos. Por ejemplo, las hembras *D. adjuntus* Blandford producen frontalina (1,5-dimetil-6-8-dioxabiciclo [3.2.1.] octano) (Kinzer et al., 1969) y se ha demostrado que promueve la atracción de conespecíficos, principalmente de machos (Huges et al., 1976). En otras especies, la frontalina es producida por los machos, como en el caso de *D. brevicomis* LeConte (Browne et al., 1979) y *D. ponderosa* Hopkins. En esta última especie, la función de la frontalina es múltiple, siendo atractiva en bajas concentraciones e inhibitoria en altas concentraciones (Ryker y Libbey, 1982).

Los machos pareados liberan feromonas que regulan la densidad de conespecíficos en el hospedero disuadiendo la agregación. En *D. ponderosa* y *D. psedotsugae* Hopkins se produce verbenona (4,6,6-trimetilbiciclo [3.1.1] hept-3-en-2-ona) (Pitman y Vité, 1969) que inhibe la respuesta de conespecíficos a las feromonas de agregación (Rudinsky et al., 1974). Cuando la pareja se establece cesa la liberación de feromonas disminuyendo la atracción al sitio de agregación, probablemente esto también regula la densidad de conespecíficos sobre el hospedero (Pureswaran y Sullivan, 2012).

Así los descortezadores emplean los semioquímicos para comunicarse y para mediar las interacciones intraespecíficas tales como la competencia por el sitio de establecimiento dentro del hospedero y por la pareja, ya que posterior a la agregación masiva existe entre los conespecíficos una distribución del recurso a lo largo del fuste del hospedero. Dentro del gremio de especies que coexisten en el mismo hábitat, las feromonas que son producidas por cada especie sirven para mediar la interacción interespecífica al servir como indicador de dónde (sección del fuste) y cuando (tiempo de arribo en la colonización) arribar para así repartirse el recurso (Birch et al., 1980; Lindgren y Miller, 2002).

1.1 Sistema de comunicación química de D. frontalis Zimmermann

En el reconocimiento del hospedero, las hembras de *D. frontalis* responden al α-pineno producido por las especies de pino que explota (p. ej. *Pinus oocarpa Schiede ex Schltdl.*, *P. maximinoi* H. E. Moore, *P. teocote Schiede ex Schltdl. & Cham.*). En el establecimiento de la hembra ésta produce frontalina, su función es múltiple debido a que con ella, junto con las kairomonas del hospedero, se promueve la agregación masiva de conespecíficos de ambos sexos. Pero además orienta al macho para su apareamiento (Kinzer et al., 1969; Pitman et al*.,* 1969). También produce otras feromonas con múltiple función que sinergizan o reducen la atracción a la frontalina como el *trans*-verbenol (*trans*-4,6,6-trimetilbiciclo [3.1.1] hept-3-en-2-ol) (Renwick 1967; Renwick y Vité, 1969) y mirtenol (4,6,6-trimetilbiciclo [3.1.1] hept-3-en-10-ol (Rudinsky et al., 1974). El perfil de feromonas de esta especie se complementa con las feromonas producidas por la hembra y cuando ésta se aparea con un macho el cual produce verbenona y *endo*-brevicomina (*endo*-7-etil-5-metil-6,8-dioxabiciclo [3.2.1] octano) (Renwick, 1967; Vité y Renwick, 1971; Sullivan et

al., 2007) la cual a la distancia orienta a los conespecíficos hacia el interior del foco de infestación y a corta distancia dirige a los conespecíficos hacia hospederos en proceso de colonización masiva donde hay aún producción de frontalina, es decir, hembras sin aparearse (Sullivan et al., 2007; Sullivan y Mori, 2009). La concentración de esta nube de olor en el hospedero por la liberación de feromonas de numerosas parejas promueve la búsqueda de otro hospedero.

1.2 Sistema de comunicación química de D. mesoamericanus Armendariz-Toledano&Sullivan

Debido a su reciente descripción (Armendáriz-Toledano et al., 2015) el conocimiento sobre el sistema de comunicación química de esta especie es limitado. Sullivan y colaboradores (2012) reportaron la producción de feromonas en tres instancias: 1) en hembras antes de emerger, 2) en hembras solitarias barrenando dentro del hospedero, y 3) en hembras pareadas. Los resultados reflejaron que las hembras de esta especie producen *endo*-brevicomina e ipsdienol (2-metil-6-metileno-2, 7-octadieno-4-ol) (Sullivan et al., 2012), feromonas que no son producidas por las hembras de *D. frontalis* pero si por los machos en ambas especies. No obstante, se considera que las feromonas producidas por ambos sexos de cada especie son similares. Pruebas iniciales en campo indican que la adición de *endo*-brevicomina a la feromona para atraer a *D. frontalis*, es decir, a la combinación de α-pineno y frontalina, incrementa la atracción de *D. mesoamericanus*, lo que sugiere que en esta especie la *endo*-brevicomina pudiera tener la misma función en machos buscando sitios de colonización y apareamiento (Moreno, 2008).

2. Mecanismos de aislamiento reproductivo en insectos descortezadores

La coexistencia entre especies cercanamente relacionadas implica que para poder competir por alimento y sitios de apareamiento para el desarrollo y sobrevivencia de su progenie, entre otros (Schoener, 1982; Futuyma y Peterson, 1985; Denno et al., 1995; Morris, 2011), las especies debieron evolucionar diferentes comportamientos y/o caracteres morfológicos. La divergencia en la morfología y/o el comportamiento en el uso del mismo recurso probablemente les confirie la capacidad de mantener cohesiva sus poblaciones (especie cohesiva) (Templeton, 1980) y aisladas reproductivamente entre ellas (especie biológica) (Mayr, 1963), y al mismo tiempo coexistir bajo los mismos requerimientos ecológicos. De tal forma, que dependiendo del grado de diferenciación genética que presenten las especies, la acción de la selección natural podría promover la evolución de mecanismos de aislamiento reproductivo entre especies hermanas en contra de los híbridos formados (Coyne y Orr, 2004; Bolnick y Fitzpatrick, 2007). Se han identificado diversos mecanismos de aislamiento de tipo precopulatorios (Symonds y Elgar, 2004a) y postcopulatorios (Templeton, 1980; Coyne y Orr, 2004), que operan sobre las especies en simpatría, particularmente entre las sintópicas. Los primeros evitan el encuentro entre los individuos de diferente especie a través del desarrollo de apareamientos selectivos o preferenciales positivos (Coyne et al., 2002; Bolnick, 2004; Peterson et al., 2005), la preferencia por el hábitat (Beltman y Metz, 2005; Drés y Mallet, 2002), reconocimiento de conespecíficos a través de sistemas de comunicación especializados como los olfativos y acústicos (Symonds y Elgar, 2004a; Ortiz-Barrientos et al., 2004; Smadja y Butlin, 2009) y la alocronía en el apareamiento (Friesen et al., 2005). Los segundos operan para evitar el desarrollo de descendencia una vez que ha ocurrido la cópula, entre ellos destacan la incompatibilidad de la genitalia, eliminación del esperma (Price et al., 2001; Servedio, 2001) inviabilidad, infertilidad y esterilidad que causan mortalidad de los cigotos, o híbridos incapaces de producir descendencia fértil (Orr y Turelli, 2001; Tao y Hartl, 2003).

En insectos descortezadores los mecanismos de aislamiento reproductivo son particularmente referidos a aquellos relacionados para evadir el encuentro y la cópula entre individuos de diferentes especies. Los mecanismos que destacan son la estratificación del hábitat y del hospedero, la alocronía en los periodos de vuelo o en actividades biológicas, producción diferencial de feromonas y compuestos sinergistas e inhibidores, preferencia del hospedero, umbral de tolerancia a la toxicidad de la resina del hospedero, sistema de cortejo como la estridulación (Sturgeon y Mitton, 1982). Estudios sobre cruzas forzadas entre especies cercanamente relacionadas han resultado en una producción reducida significativamente de híbridos a lo que se ha inferido sobre posibles mecanismos postcopulatorios incompletos (Zúñiga et al., 1995). Sin embargo hasta la fecha no existe ni un estudio que describa formalmente los mecanismos de aislamiento reproductivo entre especies hermanas de *Dendroctonus* con los mismos requerimientos ecológicos.

Dentro de las especies de *Dendroctonus* la producción de feromonas resulta ser poco diferenciada dificultando su estudio filogenético y se ha sugerido que la especificidad que las especies muestran hacia las feromonas no debe ser interpretado como el único mecanismo de aislamiento reproductivo (Lanier y Burkholder, 1974; Symonds y Elgar, 2004b).

3. Coexistencia entre las especies del género Dendroctonus

Las especies del genero *Dendroctonus* son consideradas especialistas en relación a la explotación de pinaceas de un un solo género, *Pinus* L (Salinas-Moreno et al., 2004). La diversificación de las especies de descortezador de este género, ha resultado en una repartición del recurso y coexistencia a diferentes niveles geográficos (Zúñiga et al., 1999). En alopatría las especies pueden explotar la misma o diferentes especies de hospedero.

En simpatría las especies coexisten explotando diferentes especies de pino y/o a diferentes altitudes (parapátrica). La coexistencia en sintopía (especies hermanas que presentan sobrelapamiento geográfico y ocupan el mismo hábitat) (Rivas, 1964), es más compleja debido a que varias especies coexisten en un mismo hospedero, lo cual puede darse con especies de descortezador con lejana relación filogenética y con diferentes funciones ecológicas. Esto último se refiere a la coexistencia entre especies que arriban primero en hospederos susceptibles, responsables de promover la muerte del hospedero (especies primarias) y entre especies que después del establecimiento de las especies primarias que han minimizado las defensas del árbol arriban colonizando las secciones del fuste aún no utilizadas (especies secundarias) y cuya función es contribuir con la degradación del recurso (Svihra et al., 1980).

Las especies del género *Dendroctonus* han sido agrupadas en cinco complejos en función de su similitud morfológica y atributos biológicos (Wood, 1982; Lanier et al., 1988). Uno de esos complejos es el llamado "*Dendroctonus frontalis"*. El complejo incluye las especies, *D. adjuntus, D. approximatus* Dietz, *D. brevicomis, D. frontalis*, *D. mexicanus* Hopkins, y *D. vitei* Wood (Lanier et al., 1988) y a una especie de reciénte descripción, *D. mesoamericanus* (Armendariz-Toledano et al., 2015). La coocurrencia en un mismo huésped por múltiples especies del complejo es común y ampliamente conocida en este grupo de insectos (Wood, 1982; Zúñiga et al., 1995; Moser et al., 2005). Sin embargo se ha planteado con base en la teoría de la competencia (Denno et al., 1995; Amarasekare, 2002), que es poco probable que estas especies puedan existir en espacio y tiempo en un mismo huésped, sin la evolución de mecanismos que las mantenga separadas cómo especies, observándose así un comportamiento de ataque inicial y agresivo por especies primarias, seguido de especies secundarias que son menos agresivas, las cuales

regularmente colonizan espacios del huésped que las primarias no ocuparon inicialmente. Tal aparente repartición del nicho ocurre cuando especies no agresivas, como *D. approximatus,* colonizan la base del hospedero cuyo fuste ha sido colonizado previamente por una especie primaria, por ejemplo *D. mexicanus* (Zúñiga et al., 1999). Sin embargo la coocurrencia múltiple entre especies agresivas en un mismo huésped y la repartición del nicho entre ellas es menos evidente. Pocos ejemplos pueden citarse. *D. frontalis y D. mexicanus*, dos especies hermanas, coexisten en la Sierra Gorda del Estado de Querétaro, México sobre *Pinus pseudostrobus* Lindley (Zúñiga et al., 1995) y en Arizona, EUA en rodales de *P. leiophylla* Schiede y Deppe (Moser et al., 2005). Igualmente, se ha reportado la coexistencia de *D. frontalis* con *D. brevicomis* sobre *P. ponderosa* Douglas en Arizona, EUA (Davis y Hofstetter, 2009), y con *D. vitei* en el sureste de México, Guatemala y Honduras; y también a *D. mexicanus* y *D. adjunctus (*Zúñiga, per. com.) sobre *Pinus hartwegii* Lindley en el estado de México, México.

3.1 Coexistencia entre D. frontalis y D. mesoamericanus

D. mesoamericanus es una especie de reciénte descripción y fue observada por primera vez en una infestación forestal que se presentó en Belice entre los años 2000 y 2002 (Haack et. al., 2000; Midtgaard y Thunes, 2002; Billings et al., 2004). A esta especie se le encontró cohabitando el mismo hospedero con *D. frontalis*. Posteriormente fue registrada en Chiapas y otras regiones del sureste de México y Centroamérica (Niño-Domínguez, 2007; Moreno, 2008; Armendáriz-Toledano et al., 2015). Análisis filogenéticos usando el gen citocromo oxidasa I (COI) indicaron que *D. frontalis y D. mesoamericanus* forman un clado monofilético y sugieren que estas especies divergen entre sí como un evento reciente (Armendariz-Toledano, 2015). Adicionalmente, una diferencia en el número

cromosómico entre las especies sugiere aislamiento reproductivo completo (Zúñiga, per com.). No obstante la formación de cruzas en laboratorio mostraron que hay transferencia espermática y desarrollo de larvas híbridas por lo menos del primer estadio (Armendariz-Toledano, 2014), mientras que en estudios preliminares en campo (Niño-Domínguez, datos sin publicar) no se han encontrado parejas heteroespecíficas en árboles colonizados por ambas especies, lo cual sugiere que debe haber mecanismos de aislamiento reproductivo de tipo precopulatorio más eficiente antes del apareamiento.

Por otra parte, se ha observado que *D. mesoamericanus* presenta atributos morfológicos, biológicos y ecológicos similares a las especies que integran el complejo frontalis, que la relaciona como una especie hermana de *D. frontalis*, lo cual sugiere que esta nueva especie también podría desarrollar comportamientos agresivos. No obstante, desde un punto de vista ecológico y evolutivo surge la pregunta sobre cómo dos especies hermanas con requerimientos ecológicos similares, mantienen sus poblaciones aisladas una de la otra sin la producción de híbridos.

Estudios preliminares sugieren que el contacto entre las especies (y por lo tanto la posibilidad de formar híbridos) puede ser evadido por la repartición espacial del hospedero y por presentar diferentes tiempos de arribo. Se ha observado a la progenie de cada especie emerger de manera segregada en el espacio y en el tiempo. Espacialmente, *D. frontalis* emerge por arriba de los 2 m a partir del suelo, mientras que *D. mesoamericanus* emerge por debajo de esta altura. Temporalmente los adultos de cada especie emergen alocrónicamente, es decir, *D. frontalis* emergen primero que *D. mesoamericanus*, aunque no se sabe cuál es la duración en el desarrollo de la progenie de cada especie, ni el tiempo de arribo de cada una, que pudiera explicar este comportamiento. *D. mesoamericanus* emerge entre 5 y 15 días después de haberse iniciado la emergencia de *D. frontalis*.

Justificación

Las especies de descortezador presentan un comportamiento de cooperación entre los conespecíficos para la obtención del recurso a través de la agregación masiva que ocasiona la muerte del hospedero, con una posterior segregación de los conespecificos para repartirse el recurso espacialmente. Sin embargo algunos modelos predicen que tal cooperación puede darse inclusive entre especies primarias que explotan el mismo recurso cuando la población de ambas especies no alcanza la densidad necesaria para abatir las defensas del hospedero (Okland et al., 2009). La cooperación entre diferentes especies con similar comportamiento en la obtención del recurso podría ser altamente perjudicial para las comunidades de pino, como lo sugiere la infestación devastadora que ocurrió en Belice, donde un gran porcentaje de la cobertura forestal fue eliminada por este fenómeno y donde fueron encontradas como agentes causales a *D. frontalis y D. mesoamericanus* (Midtgaard y Thunes, 2002)

El estudio del sistema de comunicación química que actualmente existe sobre los insectos descortezadores obedece al hecho de que el uso de semioquímicos representa una herramienta no destructiva y altamente específica para el manejo de los descortezadores. Sin embargo, la gran mayoría de estos estudios se enfocan a una especie en particular. En un fenómeno como lo es la coexistencia de dos especies cercanamente relacionadas que explotan el mismo recurso es importante conocer su sistema de comunicación química y cómo operan para permitir su actual coexistencia. Entender su sistema de comunicación química en la obtención de nuevos hospederos, pudiera representar una herramienta importante en el monitoreo y manejo de las poblaciones de ambas especies en las zonas donde esta interacción se presenta.

Cómo en todo trabajo pionero se requiere de información básica que permita establecer las directrices de futuros estudios, en este caso las preguntas inherentes a la coexistencia entre *D. frontalis* y *D. mesoamericanus* fueron ¿Cómo éstas dos especies logran coexistir en sintopía siendo especies cercanamente relacionadas? y siendo los semioquímicos un sistema de comunicación importante entre las especies de descortezadores, ¿es posible que modulen su aislamiento reproductivo?

La consecuente formulación de la hipótesis toma en cuenta tres aspectos importantes observados entre *D. frontalis* y *D. mesoamericanus*: 1) Existe repartición espacial del recurso a lo largo del fuste del hospedero. 2) De manera natural, no se encuentran emparejamientos entre las especies, aún con la obtención de estadios tempranos a través de intercruzas bajo condiciones de laboratorio, y 3) Las hembras de ambas especies producen una mezcla de feromonas diferencial.

Hipótesis general

Si *D. frontalis* y *D. mesoamericanus* coexisten sobre el mismo hospedero y las hembras de cada especie producen un perfil de semioquímicos diferente, entonces estos deberán mediar el aislamiento reproductivo precopulatorio entre estas dos especies.

Objetivo general

Registrar evidencia comportamental relacionada a la discriminación de machos a los semioquímicos producidos por hembras de ambas especies de descortezador, como un indicador de reconocimiento de la pareja que pueda ser interpretado como mecanismo de aislamiento reproductivo.

Objetivos particulares

1. Determinar en laboratorio si los semioquímicos producidos por las hembras de cada especie permiten a los machos la discriminación de la pareja.

2. Identificar en laboratorio las feromonas que regulan el comportamiento de discriminación de los machos para el reconocimiento de la pareja.

3. Registrar en campo la respuesta de atracción cruzada de adultos en vuelo a los semioquímicos involucrados en la discriminación de la pareja.

Materiales y Métodos generales

Sitio de estudio.- L*as* pruebas en campo se realizaron dentro de los límites del Parque Nacional Lagos de Montebello del municipio de Trinitaria, Chiapas. La vegetación predominante es de tipo pino-encino, siendo *P. maximinoi* H.E. Moore y *P. oocarpa* Schide las especies de pino dominantes. Todas las pruebas se realizaron en *P. oocarpa*, debido a que es el huésped de mayor preferencia para *D. frontalis* (Salinas-Moreno et al., 2004) y es la especie de pino más abundante dentro del Parque.

Obtención de descortezadores adultos. Los escarabajos que se utilizaron para las pruebas en laboratorio y en campo, se obtuvieron de trozas de fuste infestadas naturalmente, de los que diariamente se colectaron los adultos descortezadores emergidos y se conservaron en cajas petri con papel toalla humedecida, a 10 ºC. La identificación de las especies se realizó en primer lugar segregando los adultos con presencia (*D. mesoamericanus*) o ausencia (*D. frontalis*) de estrías en la parte pre-episternal del protórax. Posteriormente cada grupo se segregó en sexos. En *D. frontalis* las hembras se reconocieron por la presencia de un abultamiento sobresaliente en la parte antero-lateral del protórax (micangia), que está ausente en machos, éstos además se reconocieron por

presentar tubérculos prominentes en la parte frontal de la cabeza. En *D. mesoamericanus* los sexos se distinguieron por la presencia de un discreto abultamiento de la micangia en hembras y que esta ausente en machos, y a través de observar una leve elevación frontal de los tubérculos en hembras, pero prominentes en machos.

Los capítulos siguientes representan los trabajos realizados para la comprobación de la hipótesis general y que derivaron en artículos publicados, sometidos o en preparación. Los capítulos II y III presentan evidencia sobre la respuesta de los machos de cada especie para el reconocimiento de la pareja mediante el reconocimiento de semioquímicos relacionados a las hembras de cada especie, así como también se presenta el perfil de volátiles antenalmente activos para los machos y que están relacionados a los semioquímicos producidos por hembras de cada especie. Además se presenta evidencia de dos feromonas producidas por las hembras de *D. mesoamericanus* que modulan la respuesta de machos en la discriminación de la pareja. En el capítulo IV se presentan pruebas de atracción cruzada en campo que evidencian el grado de especificidad a los semioquímicos relacionados a las hembras de cada especie para la localización del hospedero y la búsqueda de la pareja. En el capítulo V y último, se discute de manera general los resultados obtenidos y presentados en los artículos de los capítulos II, III y IV. La discusión es presentada desde un contexto biológico y ecológico. Finalmente se presentan las conclusiones generales de la tesis.

CAPÍTULO II

Pheromone-Mediated Mate Location and Discrimination by Two Syntopic Sibling Species of *Dendroctonus* Bark Beetles in Chiapas, Mexico.

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PHEROMONE-MEDIATED MATE LOCATION AND DISCRIMINATION BY TWO SYNTOPIC SIBLING SPECIES OF *Dendroctonus* BARK BEETLES IN CHIAPAS, MEXICO

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ABSTRACT

Where their geographic and host ranges overlap, multiple sibling species of tree-killing bark beetles may simultaneously attack and reproduce on the same hosts. However, sustainability of these potentially mutually-beneficial associations demands effective prezygotic reproductive isolation mechanisms between the interacting species. The aggressive pine bark beetle *Dendroctonus frontalis* Zimmermann is syntopic in the Central American region with a recently-described sibling species, *Dendroctonus mesoamericanus* Armendáriz-Toledano and Sullivan, and mechanisms for their reproductive isolation are uncertain. We therefore investigated whether semiochemicals mediate species discrimination by mate-seeking males of either species. In olfactometer bioassays, walking males of both species strongly preferred odors from gallery entrances of conspecific females. Coupled gas chromatography-electroantennographic detection (GC-EAD) and gas chromatography-mass spectrometry (GC-MS) discovered sixteen different olfactory stimulants for males in these odors, but only two, ipsdienol and *endo*-brevicomin (both from *D. mesoamericanus* females), differed significantly in quantity between female-associated odors of either species. In olfactometer bioassays with either 10, 1, or 0.1 female entrance-equivalents of synthetic semiochemicals, the combination of ipsdienol and *endo*-brevicomin inhibited responses of male *D. frontalis* and enhanced responses of male *D. mesoamericanus* to a pair of compounds associated with female entrances of both species (i.e., the pheromone component frontalin and host odor *alpha*-pinene). We conclude that ipsdienol and *endo*-brevicomin, pheromone components produced by females of just one of the two species (i.e., *D. mesoamericanus*), mediate interspecific mate discrimination by males of both species and provide an apparently symmetrical reproductive isolation mechanism.

Key Words- Coevolution, bark beetle, walking bioassay, short-range attraction, reproductive isolation mechanisms, pheromone.

INTRODUCTION

Bark beetles (Coleoptera: Curculionidae: Scolytinae) feed and reproduce primarily within the phloem of trees and include some of the most significant biotic mortality agents of trees worldwide. The majority of species colonize only dead, diseased, or severely weakened hosts (Raffa et al. 1993), however a minority of species, particularly in the genera *Scolytus* Geofroy, *Dendroctonus* Erichson, and *Ips* DeGeer, can colonize and kill healthy trees (S. L. Wood 1982). Successful colonization of vigorous trees by these aggressive bark beetle species relies on group release of aggregation pheromones that mediate synchronous attacks in numbers (typically thousands of individuals) sufficient to overwhelm host defenses which otherwise would kill or expel smaller numbers of invaders (Raffa and Berryman 1983; Seybold et al. 2006). The bark beetle *Dendroctonus frontalis* Zimmermann is a major mortality agent of *Pinus* L. that ranges throughout the southeastern United States of America (USA) and from Arizona, USA south to Nicaragua (Clarke and Nowak 2010; Billings et al. 2004). It has been found existing in apparent syntopy with certain other primary *Dendroctonus* species where their distributions overlap (S. L. Wood 1982; Zúñiga et al. 1995; Moser et al. 2005, Davis and Hofstetter 2009). Within the Central American region *D. frontalis* has been observed to attack trees apparently simultaneously with *Dendroctonus mesoamericanus* Armendáriz-Toledano and Sullivan, a newly-recognized sibling species (Armendáriz-Toledano et al. 2015). Prior to its recognition as a distinct species, *D. mesoamericanus* was synonymous with *D. frontalis*. Arrivals of flying beetles and attacks by either species tend to be concentrated on different sections of host

bole but have substantial overlap in time and space such that entry sites and galleries are intermixed (Moreno 2008).

Since colonization success requires sufficient abundance of attacking beetles to deplete the host's defenses, there may be selective benefits to joint attack by multiple primary bark beetle species when local abundance of any single species is insufficient (Okland et al. 2009). Consequently, the negative effects of competition between species may be outweighed by the greater host availability that is rendered by communal mass attack (Svihra et al. 1980; Wagner et al. 1985; Ayres et al. 2001; Okland et al. 2009). This hypothesis is supported by evidence that the aggregation pheromones of syntopic, primary *Dendroctonus* species can be cross-attractive (Gaylord et al. 2006; Hofstetter et al. 2008). There is at least some degree of attraction by flying *D. frontalis* and *D. mesoamericanus* to blends of mutually-produced pheromone components, although low responses by *D. mesoamericanus* to synthetic lures in trapping trials suggest that the aggregation-mediating semiochemicals for this species have not yet been fully characterized (Sullivan et al., 2012).

Pheromones function in many insects to confer premating reproductive isolation between species, and such has been proposed for bark beetles (Lanier and Wood 1975; D. L. Wood 1982; Sturgeon and Mitton 1982; Raffa 2001). The intermixing of closely related species that presumably occurs during joint mass attacks by bark beetles should promote the evolution and persistence of effective pre-mating reproductive isolation mechanisms (Pfening 2012). Otherwise, unproductive interspecific sexual interactions might consume time and energy resources of individual beetles and reduce their lifetime reproductive capacity (Gröning and Hochkirch 2008). Furthermore, the greater lengths of time that males would be exposed on the bark surface in pursuing and rejecting (or being rejected by) heterospecific females would render them more susceptible to predation (Bunt et al.

1980). However, evidence of species cross-attraction to pheromones and stronger overlap in pheromone blend composition among sympatric bark beetle species has created uncertainty about the importance of pheromones in reproductive isolation in bark beetles (Lanier and Burkholder 1974; Symonds and Elgar 2004).

In laboratory crossing studies, confinement of males of *D. frontalis* and *D. mesoamericanus* over gallery entrances of heterospecific females resulted in pairing, sperm transfer, gallery formation, egg laying, and larval development, although measures of pairing success such as frequency of sperm transfer, gallery length, and brood production were generally less in heterospecific than conspecific pairings (Armendáriz -Toledano et al. 2014). Hybrid viability and fertility have not been tested because the authors have as yet been unable to rear brood of either conspecific or heterospecific pairings to adulthood in the laboratory. However, differing chromosome numbers between the two species suggest that post-zygotic reproductive isolation exists (Armendáriz -Toledano et al. 2014). Despite the ability to force heterospecific pairings in the laboratory, dissections of naturally-infested pines in Chiapas, Mexico have failed to reveal the presence of heterospecific pairs within zones of species overlap on the bark (Niño et al. unpublished data). These data suggest that effective premating reproductive isolation mechanisms are present that deter entry of males into gallery entrances of heterospecific females or otherwise deter pairing. Sustained coexistence of the two species and the possibility of "cooperative" mass attack behavior between them likely depend upon the existence of such mechanisms.

The objective of this work was to investigate responses by males of these two species to semiochemicals produced by both con- and heterospecific females. Previous research indicated that there are significant differences in the composition of volatiles produced by females of either species, including pheromone components for species of *Dendroctonus*

(Sullivan et al. 2012). We hypothesized that male discrimination of female pheromones could be a mechanism that deters interspecific pairings and thus reduces possible negative fitness consequences of joint mass attack by closely related species of aggressive *Dendroctonus* bark beetles.

METHODS AND MATERIALS

Biological Material. Logs (15-20 cm diam.) of both naturally-infested and uninfested, apparently healthy pines were cut throughout the year from standing *P. oocarpa* within Parque Nacional Lagunas de Montebello, Trinitaria, Chiapas, México (16° 07' N, 91° 44' W). Bark beetle adults used in bioassays were collected daily as they emerged from the infested logs enclosed in cloth bags maintained in the laboratory. Emerged adults were housed in plastic Petri plates with moistened Kimwipe® (Kimberly-Clark, Roswell, Georgia, USA) and held at 10°C. Adult beetles used in all experiments were no more than 3 d old. The ends of uninfested logs were sealed with paraffin and retained no more than ten days at 10°C prior to use in bioassays.

For bioassays, *D. frontalis* and *D. mesoamericanus* were distinguished by the presence of fine ridges on the pre-episternal area of the prothorax of *D. mesoamericanus* (Sullivan et al. 2012; Armendáriz -Toledano et al. 2015). Following bioassays, species identity of a subsample of males was confirmed through dissection and examination of genitalia (Armendáriz-Toledano et al. 2014, 2015). All research operations (insect rearing, bioassays, volatiles collections) were performed in the laboratory with an average temperature of 24°C and relative humidity of 48%. Behavioral bioassays were performed under fluorescent ceiling lighting.

Responses by Walking Males to Volatiles from Female Gallery Entrances. We assayed walking responses by individual males of each species to volatiles produced by a gallery entrance of a solitary female of either species that had been mining in an uninfested log (30 cm long x 15-20 cm diam.) for one day. Each female was confined inside an artificial pit made into the bark surface with a drill bit (3-4 mm diameter), and only a single female was infested onto any log. A disk of fine-mesh plastic screen was secured over the pit with duct-tape to prevent female escape. After 4 hr and if boring dust was apparent, the screen cover was removed and the mouth (27 mm diam.) of a modified glass aeration funnel was secured over the female entrance with a ring of paraffin wax. The wax, applied when melted, produced an air-tight seal between the bark surface and the mouth of the funnel. The funnel modification consisted of a 4 mm i.d., 30 mm-long piece of glass tubing fused with and penetrating ~10 mm into the funnel cone. Inside the cone, this tubing curved toward the center of the funnel mouth so that its opening was centered over and 5 mm distant from the gallery entrance (Supplemental 1A). By forcing air into the funnel stem and simultaneously drawing it from this tubing, it was possible to pass air directly across the gallery entrance while maintaining a closed air path. For walking bioassays we used an acrylic four-arm olfactometer (Vet et al. 1983) adapted for work with bark beetles (description and illustration given in Supplemental 1).

For odorized arms, the modified funnel attached to the infested log was interposed (i.e., connected by PTFE tubing) between the humidified/purified air supply and the olfactometer arm (Supplemental 1A). In “single odor” bioassays the test odor was delivered to only one of the four olfactometer arms selected at random whereas in “odor choice” bioassays, two opposite arms selected at random each received a different odor treatment; in both cases the remaining arms received clean air only. Treatment assignment to the four arms was re-

randomized every 4-6 trials. For the single odor bioassays there were two odor treatments (either *D. frontalis* or *D. mesoamericanus* female gallery entrances) and two subject classes (males of either species) tested in all four possible combinations. For the odor choice bioassays a single choice combination (entrances of *D. frontalis* vs *D. mesoamericanus* females) was tested with males of either species.

At the beginning of each trial, a solitary male was released on the screen covering the air outlet at the center of the olfactometer arena floor, and then the arena was immediately covered with a clear acrylic plate. During each five minute trial, we recorded whether the male crossed into any of four response circles (i.e., a 1 cm diameter circle drawn on the floor of the arena and immediately in front of the odor inlets of each arm) and the time he spent within each. If the male contacted the wall of the arena, the cover of the olfactometer was removed briefly and he was re-released at the air outlet. Males were used in a single trial and discarded. A single measure was used in statistical comparisons, namely, the time spent by each male inside the response circle (with a failure to enter any circle included in the statistical analysis as a zero-time response). Since this time was influenced both by whether or not the subject entered the response circle (i.e., located the odor source) and the time spent in the circle once entered, our 'response' measurement was an indication of two types of behaviors, namely, attraction to the odor release point and arrestment there.

Collection of Volatiles from Entrances. Immediately after behavioral bioassays were completed, volatiles from gallery entrances were sampled for 3 hr onto glass-enclosed adsorbent cartridges (117 mg Porapak-Q; SKC Inc. Eighty Four, Pennsylvania, USA). A cartridge was attached with PTFE tubing to the outlet of the aeration funnel secured over the gallery entrance (with the funnel inlet receiving purified/humidified air), and air from the funnel was drawn through the cartridge at 50 ml/min. Afterward, the cartridges were

extracted with 1.5 ml pentane (HPLC grade, Sigma-Aldrich Co., Milwaukee, Wisconsin, USA), and 3.8 µg of cycloheptanone (98%, Sigma-Aldrich) was added to each extract as an internal standard. A total of 13-14 extracts were collected (each from a different female) from gallery entrances of each species.

Electrophysiological Responses of Male Antenna. Olfactory sensitivity by male beetles to compounds within volatiles collections from female entrances was assayed by coupled gas chromatography-electroantennographic detection (GC-EAD). The GC-EAD apparatus and antennal preparation procedures were identical to those in Cano-Ramírez et al. (2012). The GC was operated with an HP-INNOwax microcapillary column (Agilent Technologies, Wilmington, Delaware, USA; polyethylene glycol phase; 30 m long, 0.25 mm diam., 25 µm film thickness) and a temperature program of 50° for 1 min, 16°/min to 80°, and then 7°/min to 200°C held 10 min. Subsamples (100 µl) from 10 to 12 of the cartridge extracts were pooled by species and concentrated approximately 10-fold passively on the lab bench. Antennae of male *D. frontalis* and *D. mesoamericanus* (11 preparations each) were tested with these concentrated volatiles extracts (2 µl into GC in splitless mode). A genuine olfactory response was recorded if an EAD deflection was detected at a particular retention time in at least four GC-EAD runs. Suspected olfactory stimulants (i.e., HID peaks coincident with EAD responses and identified by GC-MS analysis, see below) were in general confirmed in their activity by performing GC-EAD analyses with synthetic mixtures that included these suspected stimulant compounds. (Compounds without this additional step are identified in the results.)

Quantification of Olfactory Stimulants. HID peaks coinciding with EAD responses were identified by analyzing the concentrated, pooled extracts by gas chromatography-mass spectrometry (GC-MS; Hewlett-Packard model G1800C, Palo Alto, California, USA)

utilizing the same column, temperature program, and carrier flow rates as used in the GC-EAD analyses. Compound identifications were accomplished by matching both retention times and mass spectra to those of commercially-obtained compounds. The identified olfactory stimulants were then quantified in the individual (i.e., not pooled) samples derived from single aerations of female gallery entrances. Quantifications were derived from single-ion abundance ratios between the target compound and the internal standard; these ratios were then converted to ng per sample with a calibration curve produced by analyzing serial dilutions of known quantities of standard compounds.

Responses by Walking Males to Synthetic Volatile Blends. In order to identify compounds mediating species discrimination by mate-seeking males, we assayed males in the four-armed olfactometer for their responses to different synthetic combinations of volatiles. Test compounds were dissolved in hexane and released from vertically-oriented, open 5 µl microcapillaries (Drummond Scientific, Broomall, Pennsylvania, USA) with the bottom opening positioned within the airstream flowing to the inlets of each olfactometer arm (modified from Browne et al. 1974; Supplemental 2). At the beginning of each trial, capillaries were replaced with unused ones and then filled with lure solution (or pure solvent for arms not receiving a lure mixture, i.e., controls).

We investigated the behavioral effects of the two male olfactory stimulants that were produced in significantly different quantities by females of either species, namely, *endo*-brevicomin and ipsdienol. These were tested in combination with α-pinene and/or frontalin as these two odors were produced in similar quantities by female entrances of both species and have previously been identified as semiochemicals attractive to *D. frontalis* (Moreno et al. 2008; Sullivan 2011). α-Pinene was included uniformly in all blends as this host odor is normally present in association with bark beetle attacks on pines. Blends were tested in

single-odor assays with the same procedures used for tests of gallery entrance odors. Treatments were α-pinene either 1) alone 2) with frontalin, 3) with ipsdienol, 4) with *endo*-brevicomin, 5) with frontalin and ipsdienol, 6) with frontalin and *endo*-brevicomin, 7) with frontalin, ipsdienol, and *endo*-brevicomin, 8) with ipsdienol and *endo*-brevicomin (Table 1). Concentrations of compounds in the mixtures were adjusted to produce release rates from the capillaries that approximated the average release rates of the compounds measured from gallery entrances of female *D. mesoamericanus* (ipsdienol and *endo*-brevicomin) or both species (α-pinene and frontalin). Tests were also performed at one-tenth and ten-fold the single-entrance equivalent rate (Table 1). Additionally, we performed a choice assay in which opposite arms of the olfactometer received either treatment 2 or 7 at a release rate of one entrance equivalent. These treatments were chosen specifically because 2 was an approximate mimic of the odor profile of *D. frontalis* female entrances whereas treatment 7 served the same role for *D. mesoamericanus. Endo*-Brevicomin was the pure (+)-enantiomer (i.e., >99%; Sullivan et al. 2007) as produced by female *D. mesoamericanus* in the investigated population (Supplemental 3) and male *D. frontalis* in the southeastern USA (Sullivan et al. 2007). The other semiochemicals in the synthetic blends were racemic. (Our use of specific enantiomeric compositions of the semiochemicals and its implications are discussed in Supplemental 3.)

Statistical Analyses. Olfactometer assays. Behavioral bioassay data for time spent within the response circles contained an excess of zeroes and could not be transformed to meet assumptions of normality. Therefore these data were analyzed by GLM with a negative binomial distribution (Lindén and Mäntyniemi 2011) with logarithmic link function for pairwise comparisons of means. A Wilcoxon one sample test with a hypothetical median value equal to zero and a 95% confidence interval was applied to contrast between

odorized and control arms when the latter had zero counts. The overdispersion was evaluated from deviance residuals values respecting degrees of freedom of the model. For all statistical tests α=0.05. In the single-odor olfactometer bioassays, we tested whether males were arrested by an odor source or synthetic blend by determining whether the time spent within the response circle significantly exceeded the average time spent within the three control circles for each trial.

For statistical analysis of quantities of individual compounds detected in entrance volatiles, compounds were first classified as either host volatiles or pheromones (Skillen et al. 1997), and then their quantities were statistically analyzed within these groupings. Data were normalized with a $\sqrt[3]{}$ (X+ 0.05) transformation and then analyzed by a one-way ANOVA. Additionally, a *t-test* was used to compare quantities of single compounds produced by females of either species. All statistical analyses were performed with SPSS Statistics Vol. 21.

RESULTS

Responses by Walking Males to Volatiles from Female Gallery Entrance. In single-odor tests with the four arm olfactometer, males of both *D. frontalis* (Figure 1A) and *D. mesoamericanus* (Figure 1B) responded significantly to odors of conspecific female entrances (i.e., spent more time at the inlet of the arm receiving odors from the female entrance than inlets of the arms receiving clean air; for *D. frontalis*: D=93.4, gl= 266; $X^2_{0.05,\ 1}$=5.1, P= 0.024; for *D. mesoamericanus*: D=19.6, gl= 238; $X^2_{0.05,\ 1}$=25.4, P< 0.001), whereas males of neither species exhibited a significant response to odors of heterospecific females (for *D. frontalis*: D= 48.4, gl=266; $X^2_{0.05,\ 1}$=2.5, P=0.112; for *D. mesoamericanus*: D= 58.3, gl= 238; $X^2_{0.05,\ 1}$=0.363, P= 0.547). Furthermore, the average time spent by *D. frontalis* males at the odor-receiving inlet was longer (approximately seven-fold) for gallery

entrance odors of female conspecifics than female heterospecifics (D=130, gl=132; $X^2_{0.05,\ 1}$=68.7, P< 0.001, Figure 1A), and similarly mean response duration by *D. mesoamericanus* males was approximately twelve times longer for odors of female conspecifics than heterospecifics (D=110, gl= 118; $X^2_{0.05,\ 1}$=12.3, P< 0.001, Figure 1B). When odors of female entrances of either species were released from opposite arms of the olfactometer (Figure 2), males of both species spent more time (greater than seven-fold on average) at the inlet receiving odor of a conspecific female entrance than the inlets receiving odor of a heterospecific female entrance or clean air (for *D. frontalis*: D=46.6, gl=157; $X^2_{0.05,\ 2}$=69.2, P<0.001; for *D. mesoamericanus*: D=61.1, gl= 129; $X^2_{0.05,\ 2}$=9.13, P<0.001).

Electrophysiological Responses of Male Antenna. At least sixteen compounds present in volatiles from gallery entrances of females of one or both species elicited responses in male antennae (Figure 3A,B; Supplemental 4). Ten different EAD responses (i.e., occurring at a specific retention time) happened invariably regardless of species of responding male or species of odor-producing female. Nine of these ten EAD responses coincided with HID peaks apparently composed of predominantly or solely single compounds: α-pinene, ß-pinene, myrcene, limonene, frontalin, linalool, longifolene, *cis*-verbenol, and verbenone. Additionally, one of these ten responses (peak 12; Figure 3A,B) was associated with an HID peak that was apparently composed of coeluting 4-allylanisole and *trans*-verbenol, as well as ipsdienol in *D. mesoamericanus* samples. A strong EAD response at the retention time of *endo*-brevicomin and a weak response at the retention time of terpinen-4-ol were stimulated in males of both species by volatiles from *D. mesoamericanus* females but not volatiles from *D. frontalis* females. A moderate response corresponding to myrtenol was produced by male *D. mesoamericanus* antennae responding to volatiles from females of either species whereas this response was weak or absent in male *D. frontalis* antennae.

Additionally, male *D. frontalis* antennae generated a weak EAD response at the retention time of an unidentified compound (peak #7) from female *D. mesoamericanus* entrances. Olfactory sensitivity by both species to α-pinene, ß-pinene, frontalin, *endo*-brevicomin, terpinen-4-ol, *cis*-verbenol, 4-allylanisole, ipsdienol, *trans*-verbenol, verbenone and myrtenol was confirmed in GC-EAD tests with synthetic mixtures.

Quantification of Olfactory Stimulants. The hydrocarbon monoterpene α-pinene, the hydrocarbon sesquiterpene longifolene, the phenylpropanoid 4-allylanisole, and several other ostensibly host-produced compounds were the most abundant olfactory stimulants in odors collected from female entrances of either species (Figure 3C). Host compounds were not isolated in significantly different quantities between odors of attacks of either species (*t-tests*; P>0.05). We quantified seven ostensibly insect-produced compounds detected by GC-EAD (figure 3D): two bicyclic ketals (frontalin and *endo*-brevicomin), and five oxygenated monoterpenes (*cis*-verbenol, ipsdienol, *trans*-verbenol, verbenone, and myrtenol). Only two such insect-produced compounds recovered from female entrances differed in quantity between species: *endo*-brevicomin (Z=2.20, P<0.001) and ipsdienol (Z=1.20, P=0.001), and these were detected only from *D. mesoamericanus* females.

Responses by Walking Males to Synthetic Volatile Blends. In single odor olfactometer assays (Figure 4), males of the two species exhibited preferences among the differing synthetic lure combinations at one tenth (for *D. frontalis*: D=127, gl=271, $X^2_{0.050,\ 7}$= 24.6, P=0.001; for *D. mesoamericanus*: D= 244, gl= 272, $X^2_{0.050,\ 7}$= 18.0, P=0.012), one (for *D. frontalis*: D= 63.7, gl= 233, $X^2_{0.050,\ 7}$= 32.4, P<0.001; for *D. mesoamericanus*: D=146, gl= 255, $X^2_{0.050,\ 7}$= 42.4, P<0.001), and ten (for *D. frontalis*: D= 78.3, gl= 272, $X^2_{0.050,\ 7}$= 65.6, P< 0.001; for *D. mesoamericanus*: D=150, gl= 272, $X^2_{0.050,\ 7}$= 38.7, P<0.001) female entrance equivalent concentrations. At the 0.1x and 10x concentrations, *D. mesoamericanus* males

responded more strongly to the complete four-component blend (the host odor α-pinene with three compounds produced by *D. mesoamericanus* females: frontalin, *endo*-brevicomin, and ipsdienol) than all odor combinations which lacked at least one of the female-produced components (Figure 4A,C). However, at the 1x concentration, elimination of ipsdienol from the four component blend did not significantly reduce male *D. mesoamericanus* response, and both this three-component lure and the complete blend were more attractive than any other tested combination (Figure 4B). Addition of frontalin to α-pinene alone did not increase responses by male *D. mesoamericanus* whereas addition of either ipsdienol or *endo*-brevicomin to α-pinene enhanced, reduced, or had no effect on responses at the three concentrations tested. Overall, the data indicate synergistic activity among all three beetle produced components for *D. mesoamericanus*.

At all three concentrations male *D. frontalis* responded more strongly to the α-pinene/frontalin combination than any other combination of components (Figure 4D, E, F). Addition of *endo*-brevicomin and/or ipsdienol to the attractive α-pinene/frontalin combination significantly reduced male *D. frontalis* responses. At the 1x and 10x concentrations, ipsdienol and the ipsdienol/*endo*-brevicomin combination reduced attraction of male *D. frontalis* to α-pinene/frontalin significantly more than did *endo*-brevicomin alone (Figure 4D, E). However, the combination of *endo*-brevicomin and ipsdienol did not reduce attraction more than ipsdienol alone at the 1x and 10 x concentrations. In general both species exhibited a lower degree of discrimination of lure combinations at the 0.1x concentration.

When males were presented a choice of two lures that each approximated the odor blend associated with gallery entrances of females of either species (for *D. frontalis* females, α-pinene/frontalin; for *D. mesoamericanus* females, α-pinene/frontalin/*endo*-brevicomin/ipsdienol; Figure 5), males of both species strongly preferred synthetic lures

approximating the odors of conspecific rather than heterospecific female entrances (for *D. frontalis*: D=59.2, gl= 237, $X^2_{0.050,\ 2}$= 54.4, P<0.001; for *D. mesoamericanus*: D= 44.1, gl=237, $X^2_{0.050,\ 2}$= 44.8, P<0.001).

DISCUSSION

Previous evidence indicates that semiochemicals produced by females of the genus *Dendroctonus* (including *D. frontalis*) mediate male location of and entry into female gallery entrances (Libbey et al. 1974; Rudinsky 1973; Rudinsky et al. 1974; McCarty et al. 1980) but also suggests that semiochemicals may not play a significant role in preventing heterospecific pairings in sibling species of *Dendroctonus* (Pajares and Lanier 1990). In our study, males of both *D. frontalis* and *D. mesoamericanus* responded to volatiles from newly-formed female gallery entrances much more strongly if the females were conspecifics rather than heterospecifics. Thus for males of these two sympatric sibling species, discrimination of the locations of females of the correct species is probably mediated by female-associated semiochemicals, and thus semiochemicals likely provide at least a partial reproductive isolation mechanism .

Among the EAD-stimulating, ostensibly beetle-produced compounds from female entrances, only two quantitatively or qualitatively distinguished odors of females of either species (i.e., *endo*-brevicomin and ipsdienol), and both were in volatiles of *D. mesoamericanus* female entrances but absent from volatiles of *D. frontalis* females. Both *endo*-brevicomin and ipsdienol are pheromone components which occur commonly in the genera *Dendroctonus* and *Ips,* respectively (Skillen et al. 1997), and they previously were reported to distinguish odors produced by mining female *D. frontalis* and *D. mesoamericanus* (Sullivan et al. 2012). The qualitative difference between species in

production of these compounds indicates their suitability as cues by which males might distinguish females of either species. Furthermore, these two compounds either individually or in combination had opposite behavioral effects on males of the two species, enhancing attraction/arrestment of *D. mesoamericanus* while inhibiting that of *D. frontalis.* These changes occurred when these compounds were added to the lure combination of *alpha*-pinene and frontalin, components produced in roughly similar quantities by female entrances of both species. Our tests with synthetic mixtures (Figure 4) indicate that both compounds of the pair contribute to reducing cross-attraction since (1) elimination of ipsdienol from the four component blend invariably (i.e., across all concentrations tested) increased attraction of *D. frontalis*, whereas (2) elimination of *endo*-brevicomin invariably reduced the attractiveness of the four component lure combination to *D. mesoamericanus.* Frontalin was evidently a key component of the attractant for both species as elimination of frontalin reduced responses to the most attractive combination for each respective species (i.e., frontalin and α-pinene for *D. frontalis* and the complete four-component blend for *D. mesoamericanus*).

The degree of male discrimination of odors in choice bioassays was similar whether the odors consisted of volatiles from female entrances of either species (Figure 2) or synthetic mixtures differing solely in the presence *endo*-brevicomin and ipsdienol in approximately the same concentrations as produced by *D. mesoamericanus* female gallery entrances (Figure 5). Although additional semiochemicals may be involved, our data imply that ipsdienol and *endo*-brevicomin from female *D. mesoamericanus* were the cues that produced the observed discrimination by males of odors of female entrances of either species. However, the reader should note that the ipsdienol of our tests was racemic, whereas female *D.*

mesoamericanus were found to produce >95% (+)-ipsdienol (Supplemental 3); this difference could have affected the outcome of our tests.

Although *endo*-brevicomin is not produced by *D. frontalis* females, it is produced by *D. frontalis* males both before and after pairing with a female (Vité and Renwick, 1971; Sullivan et al. 2007, 2012), and ipsdienol also may be produced by some *D. frontalis* males (Sullivan et al. 2012). Rudinsky et al. (1974) found that *endo*-brevicomin inhibited arrestment of walking male *D. frontalis* by female-associated odors and induced them to produce their "rivalry chirp" associated with male-male encounters. Thus in the context of intraspecific interactions (i.e., as a pheromone), *endo*-brevicomin apparently signals to walking *D. frontalis* males that a gallery entrance already contains a paired female and is thus not suitable for entry. Our new data therefore imply that *endo*-brevicomin additionally functions in an interspecific context as a kairomone or synomone by deterring male *D. frontalis* from entering gallery entrances of syntopic *D. mesoamericanus* females. Such dual functionality of pheromones as kairomones that mediate interspecific interactions is common in bark beetles (Byers 1989) and is an example of semiochemical parsimony (Blum 1996). However, since male *D. frontalis* apparently produce key components of the *D. mesoamericanus* female pheromone that are lacking in *D. frontalis* females (i.e., *endo*-brevicomin and possibly also ipsdienol), our data suggest that entrances with *D. frontalis* pairs may be attractive to *D. mesoamericanus* males.

In addition to frontalin, *endo*-brevicomin, and ipsdienol, four more compounds which have been demonstrated to function as pheromone components in the genus *Dendroctonus* (Skillen et al. 1997) and that produced EAD responses in both species were detected in female gallery entrances: *cis*-verbenol, *trans*-verbenol, verbenone, and myrtenol. All four were detected from both *D. frontalis* and *D. mesoamericanus* female entrances and in a

previous study were isolated directly from female adults of both species in Chiapas (Sullivan 2011). The quantities detected from female entrances likely arose both from the beetles themselves and from host-released α-pinene being oxidized either spontaneously or by the beetles' microbial associates (Hughes 1973; Renwick et al. 1973; Seybold et al. 2006). There is evidence that these compounds are pheromone components for *D. frontalis* (Sullivan 2011) and may affect close-range behaviors of mate seeking males. Both verbenone and myrtenol apparently influence acceptance of gallery entrances by male *D. frontalis* and may, like *endo*-brevicomin, mediate their avoidance of entrances already occupied by a male (Rudinsky 1973; Rudinsky et al. 1974). However, in the present study there was not a significant difference in the concentrations of these compounds associated with female gallery entrances of either species (despite a broad trend toward greater quantities in association with *D. mesoamericanus*), hence it is unlikely these compounds play a role in species discrimination by males. For this reason they were not included in our tested synthetic lure mixtures, although they merit additional future study for a possible role in mediating interactions between the two species.

The apparent absence of heterospecific pairings in nature suggests that pre-mating reproductive isolation between *D. frontalis* and *D. mesoamericanus* is quite complete, and it is likely that the semiochemical mechanisms examined in our study are complemented by mechanisms involving other senses and types of behaviors. Both *Dendroctonus* and *Ips* bark beetles stridulate during interactions within and between sexes and these acoustic cues apparently mediate pairing to some extent (Ryker 1988). Evidence of taxonomically distinct sound patterns has suggested a role for acoustic cues in reproductive isolation in bark beetles (Michael and Rudinsky 1972; Rudinsky and Michael 1973), however there is as yet no direct evidence for this function (Lewis and Cane, 1992). Partitioning of the host

bole (albeit incompletely) between *D. frontalis* and *D. mesoamericanus* (Moreno 2008) and a possible difference in timing of peak arrival on the host likely reduce the amount of direct, cross-species interaction between the sexes and likely reduce opportunities for interspecific pairings. Differences in host species preferences, if they exist, might have a similar effect (Lanier and Burkholder 1974). In addition, differences in composition of the aggregation pheromone (which affects flying individuals of both sexes and may include contributions of pheromone components by both sexes, as is true for *D. frontalis*) may enhance spatial/temporal separation of species (Symonds and Elgar 2004). However, it is not yet apparent whether aggregation pheromone compositions differ for *D. frontalis* and *D. mesoamericanus* (Sullivan et al. 2012) and this is a topic of ongoing investigation.

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REFERENCES

Armendáriz-Toledano F, Niño A, Sullivan BT, Macias-Samano J, Victor J, Clarke SR, Zuñiga G (2014) Two species within *Dendroctonus frontalis* (Coleoptera: Curculionidae): evidence

from morphological, karyological, molecular, and crossing studies. Ann Entomol Soc Am 107:11-27

Armendáriz-Toledano F, Niño A, Sullivan B T, Kirkendall LR, Zúñiga G (2015) A new species of bark beetle, *Dendroctonus mesoamericanus* sp. nov. (Curculionidae: Scolytinae) in southern Mexico and Central America. Ann Entomol Soc Am, published online.

Ayres BD, Ayres MP, Abahamson MD, Teale SA (2001) Resource partitioning and overlap in three sympatric species of *Ips* bark beetles (Coleoptera: Scolytidae). Oecologia 128:443-453

Billings RF (1985) Southern pine bark beetles and associated insects: Effects of rapidly-released host volatiles on response to aggregation pheromones. J Appl Entomol 99:483-491

Billings RF, Clarke SR, Espino-Mendoza V, Cordón-Cabrera P, Meléndez-Figueroa B, Ramón-Campos J, Baeza G (2004) Gorgojo descortezador e incendios: Una combinación devastadora para los pinares de América Central. Unasylva 55:15-19

Blum MS (1996) Semiochemical parsimony in the Arthropoda. Annu Rev Entomol 41:353-374

Browne LE, Birch MC, Wood DL (1974) Novel trapping and delivery systems for airborne insect pheromones. J Insect Physiol 20:183-193

Bunt WE, Coster JE, Johnson PC (1980) Behavior of southern pine beetle on the bark of host trees during mass attack. Ann Entomol Soc Am 73:647-652

Byers JA (1989) Behavioral mechanisms involved in reducing competition in bark beetles. Holarct Ecol 12:466-476

Cano-Ramirez C, Armendáriz -Toledano F, Macias-Samano JE, Sullivan BT, Zuñiga G (2012) Electrophysiological and behavioral responses of the bark beetle *Dendroctonus rhizophagus* to volatiles from host pines and conspecifics. J Chem Ecol 38:512-534

Clarke SR, Nowak J T (2010) Southern pine beetle. USDA Forest Service Forest Insect and Disease Leaflet 49

Davis TS, Hofstetter RW (2009) Effects of gallery density and species ratio on the fitness and fecundity of two sympatric bark beetles (Coleoptera: Curculionidae). Environ Entomol 38:639-650

Gaylord ML, Kolb TE, Wallin KF, Wagner MR (2006) Seasonality and lure preference of bark beetles (Curculionidae: Scolytinae) and assocaites in a northern Arizona ponderosa pine forest. Environ Entomol 35:37-47

Gröning J, Hochkirch A (2008) Reproductive interference between animal species. Q Rev Biol 83:257-282

Hofstetter RW, Chen Z, Gaylord ML, MCmillin JD Wagner MR (2008) Synergistic effects of *alpha*-pinene and *exo*-brevicomin on pine bark beetles and associated insects in Arizona. J Appl Entomol 132:387-397

Hughes PR (1973) *Dendroctonus*: production of pheromones and related compounds in response to host monoterpenes. Z Angew Entomol 73:294-312

Lanier GD, Burkholder WE (1974) Pheromones in speciation of Coleoptera. In: Birch MC (ed) Pheromones. North-Holland Publishing, Amsterdam, pp 161-189

Lanier GN, Wood DL (1975) Specificity of response to pheromones in the genus *Ips* (Coleoptera: Scolytidae). J Chem Ecol 1:9-23

Lewis EE, Cane JH (1992) Inefficacy of courtship stridulation as a premating ethological barrier for *Ips* bark beetles (Coleoptera: Scolytidae). Ann Entomol Soc Am 85:517-524

Libbey LM, Morgan M E, Putman TB, Rudinsky JA(1974) Pheromones released during inter- and intra-sex response of the scolytid beetle *Dendroctonus brevicomis*. J Insect Physiol 20:1667-1671

Lindén A, Mäntyniemi S (2011) Using the negative binomial distribution to model overdispersion in ecological count data. J Ecol Soc Am 92: 1414-1421

McCarty FA, Billings PM, Richerson JV, Payne TL, Edson LJ (1980) Response of the southern pine beetle to behavioral chemicals in the laboratory. J Georgia Entomol Soc 15: 307-317

Michael RR, Rudinsky JA (1972) Sound production in Scolytidae: specificity in male *Dendroctonus* beetles. J Insect Physiol 18:2189-2201

Moreno CB (2008) Diferenciación morfológica y comportamental de dos morfos de *Dendroctonus frontalis* Zimmermann (Coleoptera: Scolytinae) en Montebello, Chiapas. Thesis, El Colegio de la Frontera Sur, Chiapas, MX

Moreno B, Macías J, Sullivan BT, Clarke SR (2008) Field response of *Dendroctonus frontalis* (Coleoptera: Scolytinae) to synthetic semiochemicals in Chiapas, Mexico. J Econ Entomol 101:1821-1825

Moser JC, Fitzgibbon B, Klepzig KD (2005) The Mexican pine beetle, *Dendroctonus mexicanus*: first record in the United States and co-occurrence with the Southern pine beetle, *Dendroctonus frontalis* (Coleoptera: Scolytidae or Curculionidae: Scolytinae). Entomol News 116:235-243

Okland B, Skarpaas O, Kausrud K (2009) Threshold facilitations of interacting species. Popul Ecol 51:513-523

Pajares JA, Lanier GN (1990) Biosystematics of the turpentine beetles *Dendroctonus terebrans* and *D. valens* (Coleoptera: Scolytidae). Ann Entomol Soc Am 83:171-188

Pfening DW (2012) Evolution's wedge: competition and the origin of diversity. University of California Press. Oakland, California, USA

Raffa KF (2001) Mixed messages across multiple trophic levels: the ecology of bark beetle chemical communication systems. Chemoecology 11:49-65

Raffa KF, Berryman AA (1983) The role of host plant-resistance in the colonization behavior and ecology of bark beetles (Coleoptera, Scolytidae). Ecol Monogr 53:27-49

Raffa KF, Phillips TW, Salom SM (1993) Strategies and mechanisms of host colonization by bark beetles. In: Schowalter RD., Filip GM (eds) Beetle-pathogen interactions in conifer forests, Academic Press, London, pp 103-128

Renwick JAA, Hughes PR, Tanlentin DT (1973) Oxidation products of pinene in the bark beetle *Dendroctonus frontalis*. *J. Insect Physiol.* 19:1735-1740

Rudinsky JA (1973). Multiple functions of the southern pine beetle pheromone verbenone. Environ Entomol 2:511-514

Rudinsky JA, Michael RR (1973) Sound production in Scolytidae: stridulation by female *Dendroctonus* beetles. J Insect Physiol 19:689-705

Rudinsky JA, Morgan ME, Libbey LM, Putnam TB (1974) Antiaggregative-rivalry pheromone of the mountain pine beetle, and a new arrestant of the southern pine beetle. Environ Entomol 3:90-97

Ryker LC (1988) Acoustic studies of *Dendroctonus* bark beetles. Fla Entomol 71:447-471

Seybold SJ, Huber DPW, Lee JC, Graves AD, Bohlmann J (2006) Pine monoterpenes and pine bark beetles: A marriage of convenience for defense and chemical communication. Phytochem Rev 5:143-178

Skillen EL, Berisford CW, Camaan MA, Reardon RC (1997) Semiochemicals of forest and shade tree insects in North America and management applications. USDA Forest Service Forest Health Technology Enterprise Team Publication, FHTET 96-15

Sturgeon KB, Mitton JB (1982) Evolution of bark beetle communities. In: Mitton JB, Sturgeon KB (eds) Bark beetles in North American conifers. University of Texas Press, Austin, Texas, USA, pp 350-386

Sullivan BT, Shepherd WP, Pureswaran DS, Tashiro T, Mori K (2007) Evidence that (+)-*endo*-brevicomin is a male-produced component of the southern pine beetle aggregation pheromone. J Chem Ecol 33:1510-1527

Sullivan BT (2011) Southern pine beetle behavior and semiochemistry. In: Coulson RN, Klepzig KD (eds) Southern pine beetle II. USDA Forest Service Southern Research Station General Technical Report SRS-140, pp 25-50

Sullivan BT, Niño A, Moreno B, Brownie C, Macías-Sámano J, Clarke SR, Kirkendall LK, Zúñiga G (2012) Biochemical evidence that *Dendroctonus frontalis* consists of two sibling species in Belize and Chiapas, Mexico. Ann Entomol Soc Am 105:817-831

Svihra P, Paine TD, Birch MC (1980) Interspecific olfactory communications in southern pine beetles. Naturwissenschaften 67:518-520

Symonds MRE, Elgar MA (2004) Species overlap, speciation and the evolution of aggregation pheromones in bark beetles. Ecol Lett 7:202-212

Vet LEM, Van Lenteren JC, Heymans M, Meelis E (1983) An airflow olfactometer for measuring olfactory responses of hymenopterous parasitoids and other small insects. Physiol Entomol 8:97-106

Vité JP, Renwick JAA (1971) Inhibition of *Dendroctonus frontalis* response to frontalin by isomers of brevicomin. Naturwissenschaften 58:418-419

Wagner TL, Flamm RO, Coulson RN (1985) Strategies for cohabitation among the southern pine bark beetle species: comparisons of life-process biologies. USDA Forest Service Southern Research Station General Technical Report SO-56, pp 87-101

Wood DL (1982) The role of pheromones, kairomones, an allomones in the host selection and colonization behavior of bark beetles. Ann Rev Entomol 27:411-446

Wood SL (1982) The bark and ambrosia beetles of North of Central America (Coleoptera: Scolytidae): a taxonomic monograph. Great Basin Nat Mem 6:1-1359

Zúñiga G, Cisneros R, Salinas-Moreno Y (1995) Coexistencia de *Dendroctonus frontalis* ZIMMERMAN y *D. mexicanus* HOPKINS (Coleoptera: Scolytidae) sobre un mismo hospedero. Acta Zool Mex 64:59-62

Table 1. Composition of lure mixtures tested in walking olfactometer assays.

				Concentration (µg/µl)[c]			Approx. release rate (µg/min)[d]			Lure blend composition							
Semiochemical	Source[a]	Purity	Enant. Ratio[b]	0.1 X	1 X	10 X	0.1 X	1 X	10 X	1	2	3	4	5	6	7	8
α-pinene	Aldrich	99%	racemic	0.41	4.1	41	0.23	2.1	17	X	X	X	X	X	X	X	X
frontalin	ChemTica	>99%	racemic	0.0012	0.0121	0.121	0.00085	0.0073	0.068		X			X	X	X	
ipsdienol	Bedoukian	>93%	racemic	0.00014	0.0014	0.014	0.00006	0.00048	0.0044			X		X		X	X
endo-brevicomin	(Sullivan et al., 2007)	95%	(+)	0.00015	0.0015	0.015	0.00007	0.00060	0.0053				X		X	X	X

[a] Sigma-Aldrich Co., Milwaukee, Wisconsin, United States of America (USA); ChemTica Internacional, Heredia, Costa Rica; Bedoukian Research Inc., Danbury, Connecticut, USA.

[b] Concentration (in hexane) of components in lure solution added to release capillary.

[c] Basis for the enantiomeric ratios chosen for tests is explained in the methods text.

[d] Release rate from capillary based on quantity added to capillary, average time of loss of all liquid from capillary, and average proportions of release among compounds (determined by quantitative analysis of aerations of releasers). The 1x rate mimicked the release rate of compounds from a single gallery entrance of a female *D. mesoamericanus* (see text).

Figurecapitation

Fig. 1 Mean (±SE) time spent by walking male *D. frontalis* (A) and *D. mesoamericanus* (B) within 1 cm diam. circles located immediately in front of the inlets of a four-arm olfactometer. One arm received volatiles from a gallery entrance of either a *D. frontalis* or *D. mesoamericanus* female and the other three arms received clean air (controls). The times spent by the males at the three control arms were averaged (open bars). An asterisk indicates that arrestment time for the indicated odor treatment was significantly greater than for clean air; differing lower-case letters indicate significant differences in mean arrestment duration for odors from either species of female (GLM with negative binomial distribution and logit function, α=0.05).

Fig. 2 Mean (±SE) time spent by walking male *D. frontalis* and *D. mesoamericanus* within 1 cm diam. circles located immediately in front of the inlets of a four-arm olfactometer. Two opposite arms received volatiles from gallery entrances of either a *D. frontalis* or *D. mesoamericanus* female, and the other two olfactometer arms received clean air (controls). The times spent by the males at the two control arms were averaged (shaded bars). Within each species of male, differing lower-case letters indicate a significant difference in mean arrestment duration (GLM with negative binomial distribution and logit function, α=0.05). No male *D. mesoamericanus* entered the control circles (response of zero) and these data were excluded from the statistics.

Fig. 3 Coupled gas chromatography–electroantennographic detection (GC-EAD) analyses with the antennae of male *D. frontalis* (A) and *D. mesoamericanus* (B) exposed to pooled volatiles samples collected from gallery entrances occupied by females of either species. Bar graphs (C and D) display the average quantities (mean ± SE) of olfactory stimulants in volatiles collected from individual female entrances as determined by GC-MS. Compounds

originating ostensibly from either the host tree tissue (C) or the beetle itself (D) are displayed separately. Quantities associated with the same lower case letters were not significantly different (one-way ANOVA, α=0.05). An asterisk indicates that the quantities of a particular compound differed significantly between species (*t-test* with α=0.05). Number labels of peaks in A and B correspond to the numbering of compound identifications of C and D. HID/EAD peak 14 was composed of 2-3 coeluting compounds.

Fig. 4 Mean (±SE) arrestment time of male *D. mesoamericanus* (A-C) and *D. frontalis* (D-F) in front of the inlets of a four-arm olfactometer. A single odorized arm received synthetic combinations of volatiles α-pinene (αP), frontalin (F), ipsdienol (I), and *endo*-brevicomin (E) whereas the other three arms received clean air (controls). Results for the three control arms were averaged (filled bars). Odor concentrations were adjusted to 10 (A, D), 1 (B, E), or 0.1 (C, F) female gallery entrance equivalents (see text for additional details). An asterisk indicates that arrestment by odors was significantly greater than for controls; odor treatments labelled with the same lower-case letters did not differ significantly in mean arrestment duration (GLM with negative binomial distribution and logit function or a *Wilcoxon test* when controls had a zero response, α=0.05).

Fig. 5 Mean (±SE) time spent by walking male *D. frontalis* and *D. mesoamericanus* arrested in front of the air inlets of a four-arm olfactometer. Two opposite arms each received a single entrance equivalent concentration of a synthetic blend of volatiles associated with female entrances of either species (for *D. frontalis*: α-pinene and frontalin; for *D. mesoamericanus*: α-pinene, frontalin, ipsdienol, and *endo*-brevicomin). The times spent by the males within the response circles of the two control arms were averaged. Within each species of male, treatments labelled with different lower-case letters differed significantly in mean arrestment duration (GLM with negative distribution).

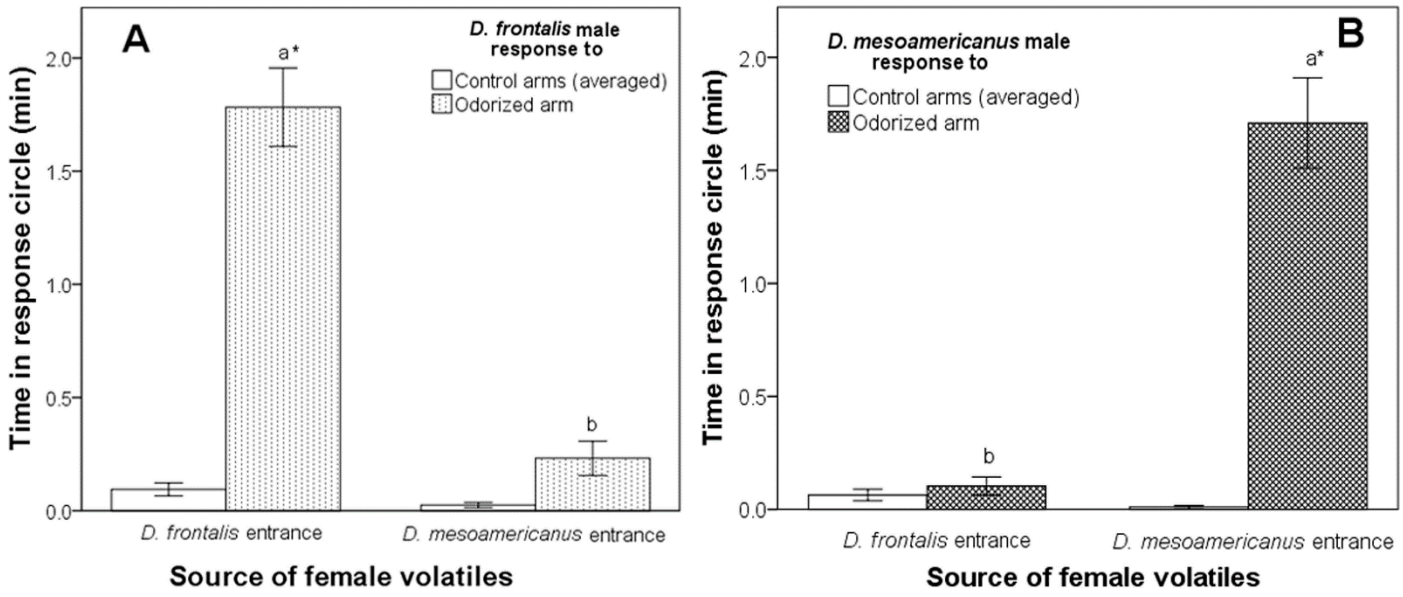

Figure 1

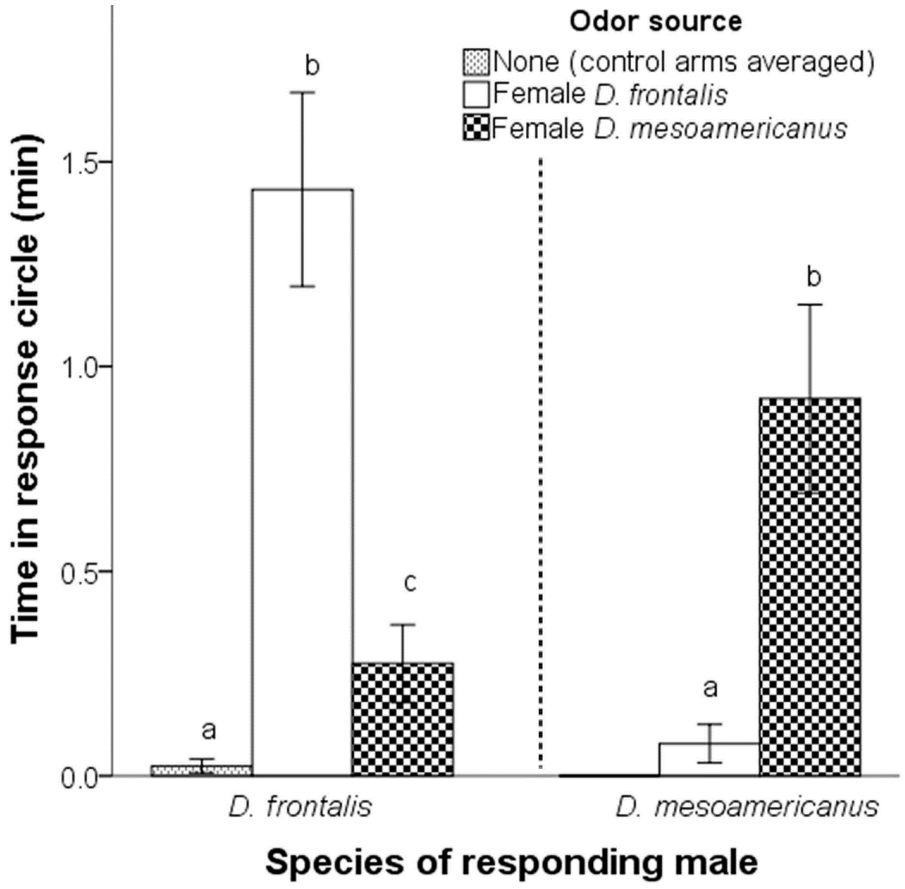

Figure 2.

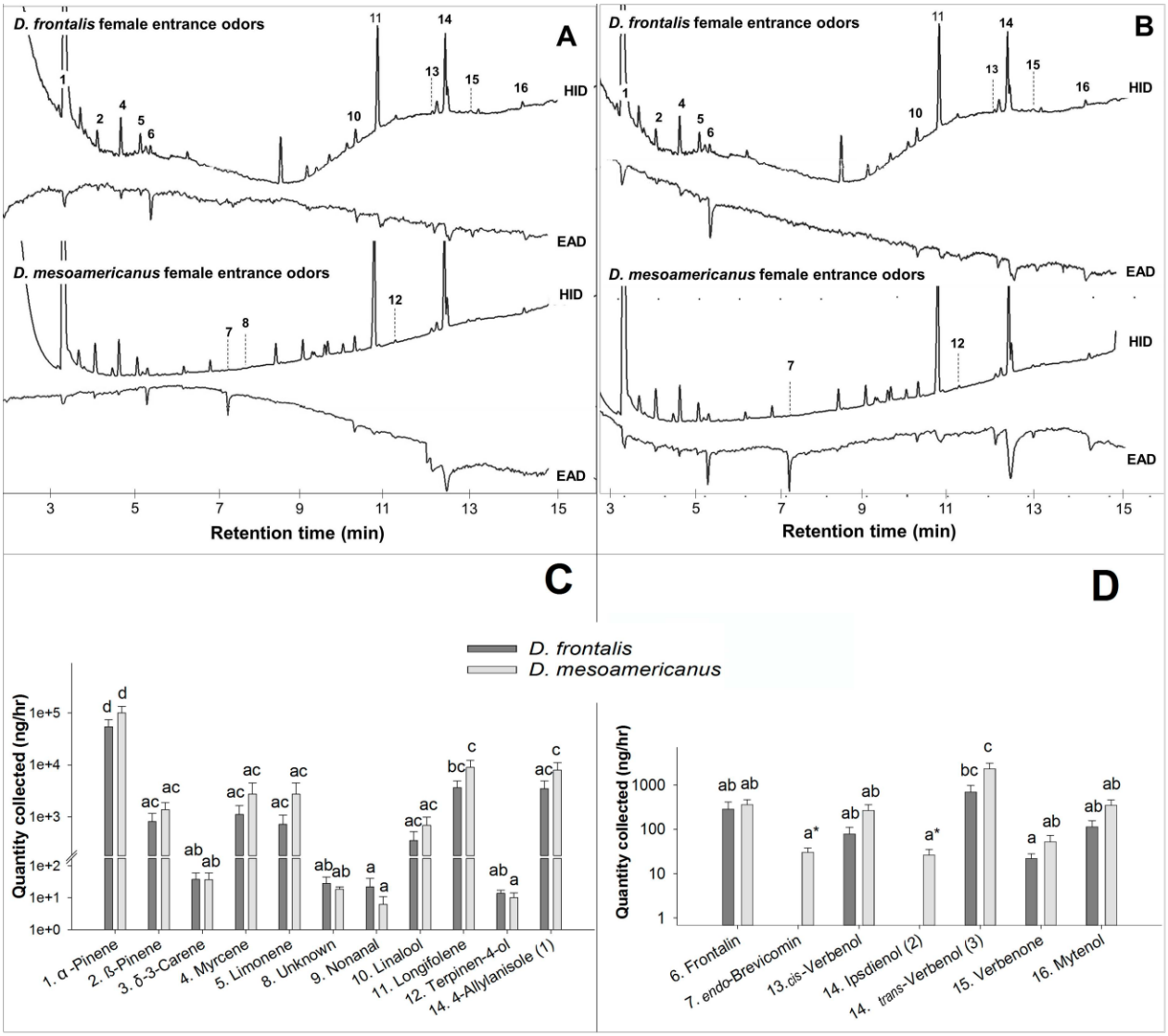
D. frontalis female entrance odors
A
HID
EAD
D. mesoamericanus female entrance odors
HID
EAD
Retention time (min)
B
D. frontalis female entrance odors
HID
EAD
D. mesoamericanus female entrance odors
HID
EAD
Retention time (min)
C
D
D. frontalis
D. mesoamericanus
Quantity collected (ng/hr)
1. α-Pinene
2. ß-Pinene
3. δ-3-Carene
4. Myrcene
5. Limonene
8. Unknown
9. Nonanal
10. Linalool
11. Longifolene
12. Terpinen-4-ol
14. 4-Allylanisole (1)
Quantity collected (ng/hr)
6. Frontalin
7. endo-Brevicomin
13. cis-Verbenol
14. Ipsdienol (2)
14. trans-Verbenol (3)
15. Verbenone
16. Mytenol

Figure 3.

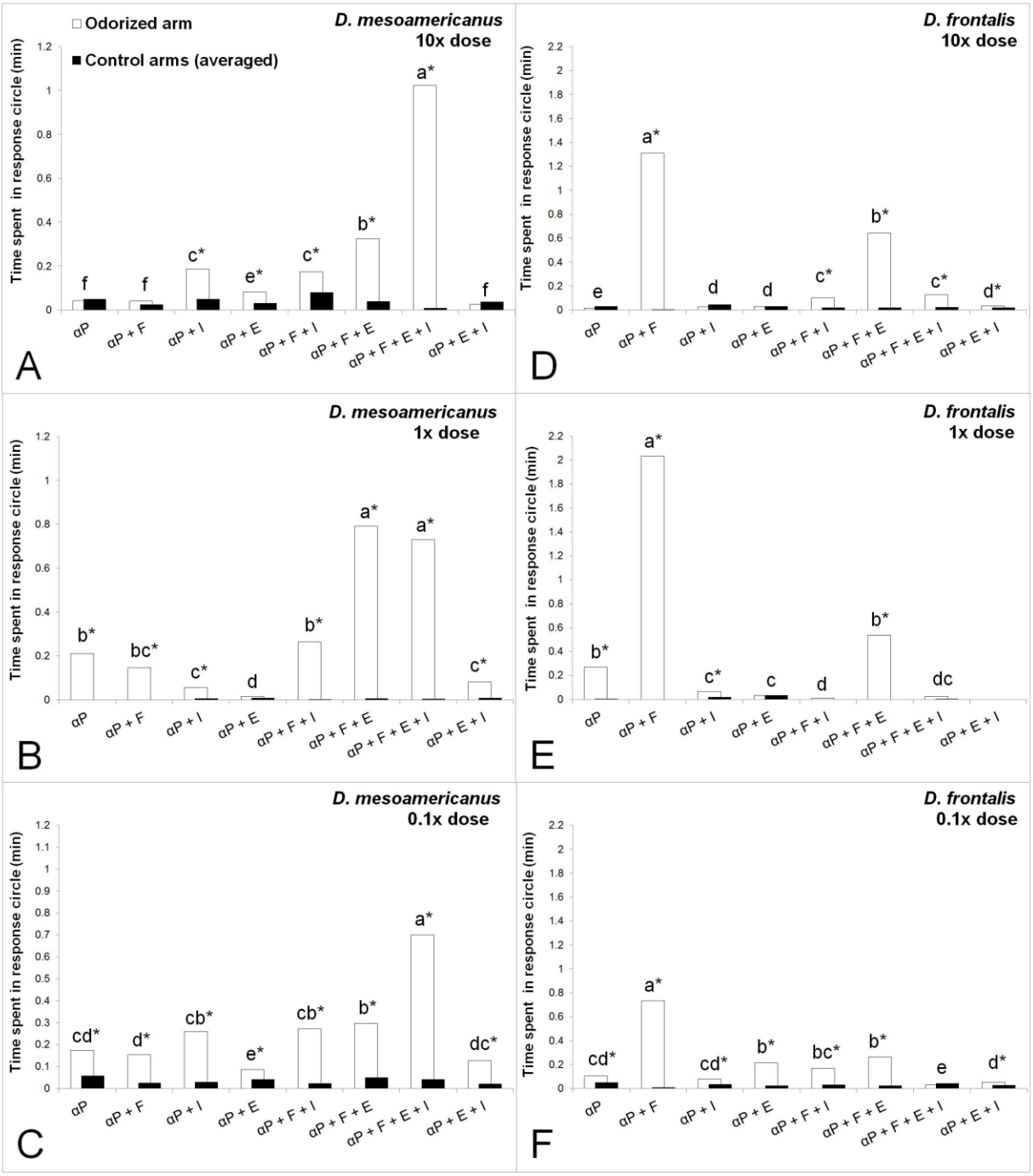
Odorized arm
Control arms (averaged)
D. mesoamericanus 10x dose
D. frontalis 10x dose
D. mesoamericanus 1x dose
D. frontalis 1x dose
D. mesoamericanus 0.1x dose
D. frontalis 0.1x dose
Time spent in response circle (min)
αP
αP + F
αP + I
αP + E
αP + F + I
αP + F + E
αP + F + E + I
αP + E + I
A
B
C
D
E
F

Figure 4.

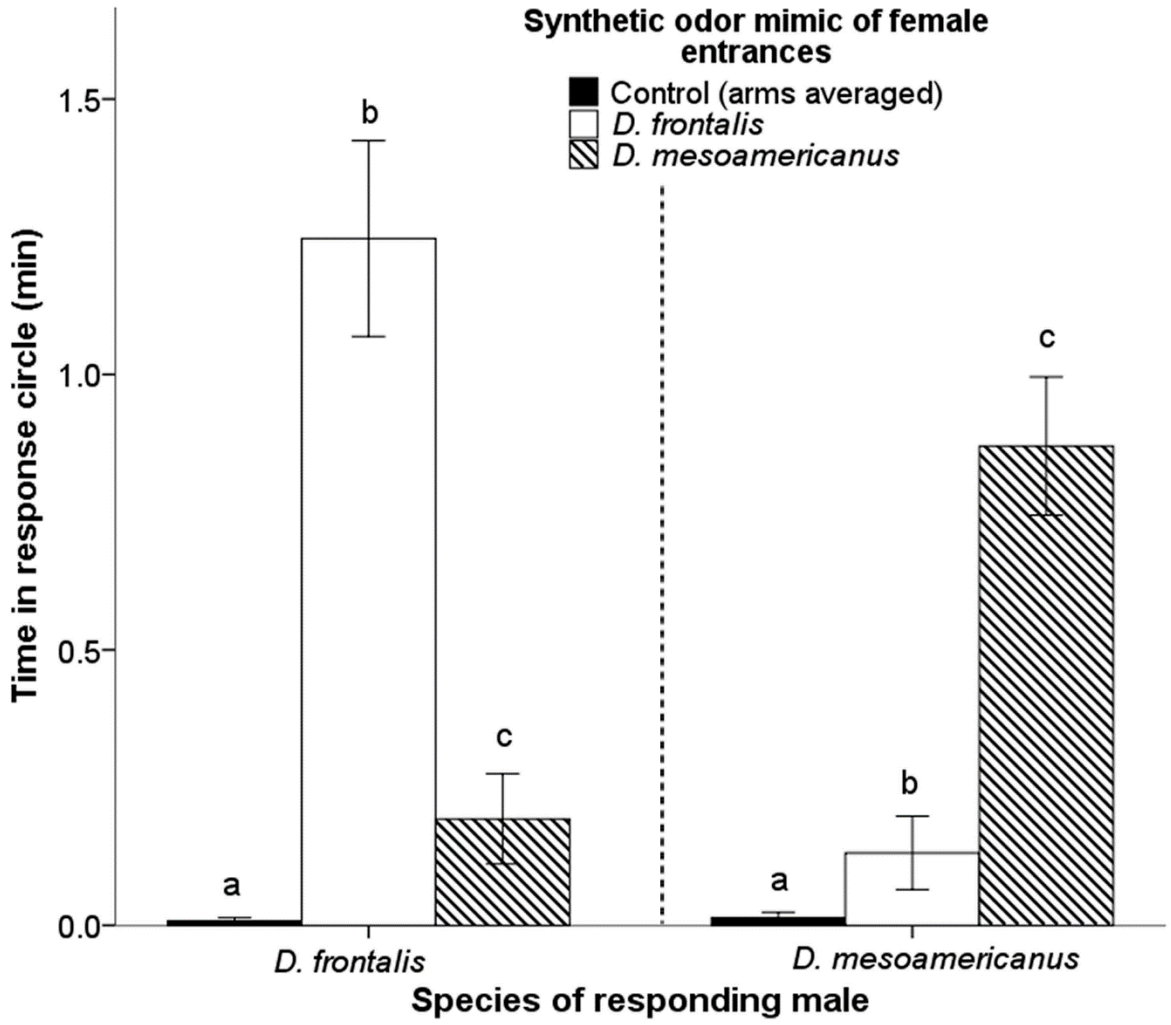
Synthetic odor mimic of female entrances
Control (arms averaged)
D. frontalis
D. mesoamericanus
Time in response circle (min)
1.5
1.0
0.5
0.0
a
b
c
a
b
c
D. frontalis
D. mesoamericanus
Species of responding male

Figure 5.

CAPÍTULO III

Frass volatiles produced by syntopic females of the sister species *D. frontalis* Zimm & *D. mesoamericanus* Toledano & Sullivan, as mediators of discriminative response of males in mate recognition.

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Artículo en preparación para su publicación.

Frass volatiles produced by syntopic females of the sister species *D. frontalis* Zimm & *D. mesoamericanus* Toledano & Sullivan, as mediators of discriminative response of males in mate recognition.

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ABSTRACT

The coexistence of *D. frontalis* y *D. mesoamericanus* on the same host is an important subject for study because of the potential for this association to increase the intensity of infestations in pine forests within the sympatric zone of these two species. Both species produce a similar semiochemical profile, however two pheromone components produced by female *D. mesoamericanus* (ipsdienol and *endo*-brevicomin) have been shown to be involved in mate species discrimination in walking and flying males. Nevertheless final mate recognition does not occur with the orientation towards colonization site and the male arrival on a host being colonized. Males must additionally explore the surface of the host to find an available female. During searching males are susceptible to predation, so they need strategies to minimize these risks by finding a female quickly. Frass expelled at the female gallery entrance contains the total semiochemical blend for attracting a male, however, it is likely that there are some attractive cues present at low concentration in the frass odor plume and sensed at close range (mm distances) but not over longer distances. It is possible that some of these minor compounds mediate interactions between species over very small distances. So the aim of this study was to test whether the frass produced by either species of bark beetle had differences in its volatile content that, when passively diffused into the immediately surrounding air, could mediate mate discrimination by males immediately outside a gallery entrance. Through laboratory tests of walking males responding to passively released frass semiochemicals associated with females of each species, it was found that the males of both species strongly discriminated between conspecific and heterospecific females. It was then verified by GC-MS analyses of frass odors that females of each species differed in the production of *endo*-brevicomin and

ipsdienol, which were produced only by female *D. mesoamericanus*. Additionally, a minor frass compound was identified as being potentially important to mate discrimination by males. In coupled gas chromatography-electroantennographic detection tests, male *D. frontalis* antennae responded to δ-3 carene, which was present in low concentrations in frass of *D. mesoamericanus* females but *not D. frontalis* females. It is possible that such low concentration compounds that distinguish odors of females of the two species could serve as short range cues to complement the longer-range mate discrimination cues (i.e., ipsdienol and *endo*-brevicomin) as they pass within very short distances of gallery entrances and associated frass.

Keywords: new species, coexistence, mate recognition, reproductive isolation.

INTRODUCTION

Dendroctonus frontalis ZIMM is a species of bark beetle that is widely distributed in the North American continent. This species is recognized as one of the main agents of mortality of pine forests throughout its range (Wood, 1982, Six and Bracewell, 2015). In the zone between the states of Michoacán, Oaxaca and Chiapas in Mexico, as well as within Guatemala, Belize, El Salvador, Honduras and, Nicaragua, it is sympatric with the newly described sibling species *D. mesoamericanus* Armendáriz-Toledano & Sullivan (Armendarís-Toledano et al, 2014; Armendáriz-Toledano et al, 2015), and both species are commonly found together in the same host trees. The syntopic coexistence among sibling species appears to be not uncommon among bark beetles (Zuñiga et al., 1999, Moser et al., 2005, Hofstetter et al., 2012, Paine et al., 1981), even though classical models of competition indicate that the probability of such syntopy is low (Denno et al. 1995). Some models predict that species of bark beetles that execute joint mass-attacks have greater

advantage in overcoming the defenses of vigorous hosts when the population density of any one species does not achieve the number of conspecifics required for successful host colonization (Okland et al., 2009).

Group attack is probably the key mechanism that allows aggressive bark beetles to secure a host resource when at high densities (Berryman et al., 1985), which implies that for coexistence of the two sibling species *D. frontalis* and *D. mesoamericanus* there must be mechanisms of interspecific communication for coordinating the mass attack, partitioning the host resource, and assuring reproductive isolation (Byers, 1989; Byers and Zhang, 2011). *Dendroctonus* have been shown to perceive their environment through visual, gustatory, acoustic, and olfactory cues (Birch, 1984, Ryker 1988, Byers, 1989, Sullivan 2011). One hypothesis regarding beetle orientation to new hosts and colonization sites is that these behaviors are mediated through beetle recognition of host kairomones or pheromones of con- or heterospecific beetles that are already established (Renwick and Vité, 1970, Wood 1982). Apparently *D. frontalis* and *D. mesoamericanus* exhibit differences in their communication system that could favor their co-existence. For example there are qualitative and quantitative differences in the production of frontalin, *endo*-brevicomin, and ipsdienol by females of either species (Sullivan et al., 2012, Niño et al., 2015), with the latter two pheromones being produced only by *D. mesoamericanus* females. However, the summed semiochemical profiles of both sexes of each species are very similar; hence volatiles produced by gallery entrances containing paired beetles may be very similar for both species during the colonization of the host (Sullivan et al., 2012).

The differential production of pheromones between the sexes of each species should correspond to an asymmetrical response to conspecific discrimination and mates (Smadja and Butlin, 2009). It has been shown in these two species that *endo*-brevicomin and

ipsdienol (when presented with frontalin and volatile host compounds) mediate the response of walking and flying males in discrimination of mates (Niño et al., 2015th 2015b). These studies probably reflect the dynamic way in which bark beetles perceive airborne semiochemicals. However more frequently dissemination of these semiochemicals occurs passively, since much pheromone release occurs after arrival on the host where beetles form pitch-tubes and release frass from which semiochemicals emanate. Apparently minor compounds that are part of semiochemical profile could play an important role in mediating interactions between species of bark beetles (Schlyter et al., 2015). Hence studies which investigate a broader range of the natural behaviors of interacting species could reveal research methodologies for studying interactions of closely related species that produce apparently similar volatile profiles during colonization, such as between *D. frontalis* and *D. mesoamericanus*.

The beetles' "frass" is a combination of resin particles mixed with beetle excrement (containing pheromone) and particles of phloem and bark that the beetle chews from the walls of the gallery during mining. Expulsion of frass from the gallery entrance is likely the major process by which pheromones are released outside the host being colonized by bark beetles (Birch, 1984; Blomquist et al, 2010). Thus extracts of this material have been used to determine the volatile profile of numerous bark beetle species (Gries et al., 1988; Paine et al 1999). Less frequently frass and its extracts have been used in behavioral studies which utilized logs being actively mined by the pioneer sex as the source of frass, and these bioassay regimes typically employed forced air to communicate odors to the test insect (a "dynamic" bioassay) (Vité and Pitman 1968 Gries et al, 1988, Paine et al 1999, Child et al, 2015). We have observed differences in the amount of frass produced by females of *D. frontalis* and *D. mesoamericanus* (on average 8.3 and 24.1 mg, respectively, unpublished

data), which coincides with larger pich-tubes observed in the field for this latter species (Armendariz-Toledano et al. 2015). This suggests that the emission of odors associated with frass and resin is higher for *D. mesoamericanus*. The total concentration of semiochemicals is one quality of an insect's olfactory signal that can encode important information, so the difference in the amount of frass produced by females of each species could have implications for mate discrimination and host partitioning. The resource used by *D. frontalis* and *D. mesoamericanus* is apparently divided into two sections along of host bole, where *D. frontalis* is concentrated in the middle and upper portions and *D. mesoamericanus* occurs in the bottom 2-3 m of the bole and larger branches of the crown (Moreno, 2008). Thus spatial partitioning of the host, qualitative and quantitative differences in chemical communication systems, and an apparent absence of heterospecific crosses in the area of overlap on the host bole (Armendaris-Toledano et al., 2014) suggest that species coexistence is mediated by mechanisms that reduce competition and allow reproductive isolation. Hence the following study was intended to investigate the behavioral responses of males of both species to volatiles passively released from frass produced by females of either species and determine the active compounds that could potentially play a role in mate discrimination by males. We hypothesize that the frass of both species presents a different composition of semiochemicals and that this allows short-range recognition by males of a conspecific female available for mating.

MATERIALS AND METHODS

Biological Material. Logs (15-20 cm diam.) of both naturally-infested and uninfested, apparently healthy pines were cut throughout the year from standing P. oocarpa within Parque Nacional Lagunas de Montebello, Trinitaria, Chiapas, México (16° 07' N, 91° 44' W).

Bark beetle adults used in bioassays were collected daily as they emerged from the infested logs enclosed in cloth bags maintained in the laboratory. Emerged adults were housed in plastic Petri plates with moistened Kimwipe® (Kimberly-Clark, Roswell, GA, USA) and held at 10°C. Adult beetles used in all experiments were no more than 3 d old. The ends of uninfested logs were sealed with paraffin and retained no more than ten days at 10°C prior to use in bioassays.

For bioassays, *D. frontalis* and *D. mesoamericanus* were distinguished by the presence of fine ridges on the pre-episternal area of the prothorax of *D. mesoamericanus* (Sullivan et al. 2012; Armendariz-Toledano et al. 2015). Following bioassays, species identity of a subsample of males was confirmed through dissection and examination of genitalia (Armendariz-Toledano et al. 2014, 2015). All insect rearing, bioassays, volatiles collections were performed in the laboratory with an average temperature of 24°C and relative humidity of 48%. Behavioral bioassays were performed under fluorescent ceiling lighting.

Responses by Males to Volatiles from Female Frass in a Permeable Platform Olfactometer. We performed assays with an olfactometer design in which odors were released by passive diffusion from the walking surface of the insect; we believed that this design might better resemble how male beetles would encounter female odors while searching the bark surface. Frass was collected from individual females of either species forced to attack bark of uninfested logs (30 cm long x 15-20 cm diam.) for one day. Each female was confined inside an artificial pit made into the bark surface with a drill bit (3-4 mm diameter; to phloem depth and at the log's midsection). Only a single female was infested onto any log. A disk of fine-mesh plastic screen was secured over the pit with duct-tape to prevent female escape. After 4 hr and if boring dust was apparent, the screen cover was removed and replaced by

the mouth of a glass vial (2 ml) in which frass expelled from the entrance accumulated. Two vials were secured over the entrance sequentially for 24 hr each. After frass collection, vials were capped and stored at -20°C for <5 d. The olfactometer (Fig. 1) consisted of an ELISA plate (polystyrene, 96 wells of 360 µl volume each, 0.7 cm well diameter, 1.2 cm depth; Corning Inc., Corning NY, United States of America, part # M0661) whose surface was covered by a rectangle of fine white screen (nylon cloth, 40 threads per cm) held taught by an acrylic frame in order to form a flat arena surface. The screen surface was divided into four equal, rectangular quadrants (4 x 6 cm; each covering 4 x 6 = 24 total wells) within the area of the plate surface, with a single well within each quadrant (B4, B9, G4, and G9; corners of a 6 x 6 well square; center of each well located 31 mm from the origin of the quadrants) designated for filling with frass (Fig. 1). Wells of quadrants not receiving frass were left empty. Frass combined from both vials of a single female entrance (approximately 16 and 42 mg of frass produced by *D. frontalis* and *D. mesoamericanus* females, respectively) was added to the designated well of one (for single odor bioassays) or two opposite (for odor choice bioassays) quadrants with both the frass source (i.e., species of female) and the quadrants receiving frass chosen at random. Frass from a single female was used for 8-12 sequential trials (40-60 min). For each such frass portion, both single-odor and odor-choice trials were performed each with two or three males of either species (tested alternately and with the first species chosen at random). First, single-odor trials were completed (i.e., with frass from a single female of either species; both species tested in random order), and then this frass was transferred to an unused ELISA plate for the odor choice tests. The ELISA plates were used once and then discarded.

During each five minute trial a single male was released at the origin of the four quadrants, and the time spent by the beetle in each quadrant was recorded. Potential visual cues from

the frass (i.e., its dark color) were not completely concealed from directly above by the screen cover but should not have been detectable from the release point of the insects.

Collection of Volatiles from Frass. Immediately after behavioral bioassays were completed, volatiles from tested odor sources were sampled for 3 hr onto glass-enclosed adsorbent cartridges (117 mg Porapak-Q; SKC Inc. Eighty Four, Pennsylvania, United States of America). For collecting frass volatiles, frass from a single ELISA well was transferred to a 12 cm long (0.4 mm diam.) piece of clean glass tubing and secured in place with glass wool plugs. One end of the frass-holding tube was connected by PTFE tubing to a source of purified nitrogen and the other end to an adsorbent cartridge. During each aeration, the frass-containing portion of the tube was secured inside an aluminum block that was heated to 60°C to enhance release of volatile compounds from the frass. Flow rate through the cartridge was 50 ml/min for both frass and gallery aerations. Afterward, the cartridges were extracted with 1.5 ml pentane (HPLC grade, Sigma-Aldrich Co., Milwaukee, Wisconsin, United States of America), and 3.8 µg of cycloheptanone (98%, Sigma-Aldrich) was added to each extract as an internal standard. A total of 12-14 extracts were collected (each from a different female) for each female species.

Electrophysiological Responses of Male Antenna. Olfactory sensitivity by male beetles to compounds within volatiles collections from females was assayed by coupled gas chromatography-electroantennographic detection (GC-EAD). The GC-EAD apparatus and antennal preparation procedures were identical to those in Cano-Ramírez et al. (2012). The GC was operated with an HP-INNOwax microcapillary column (Agilent Technologies, Wilmington, Delaware, United States of America; polyethylene glycol phase; 30 m long, 0.25 mm diam., 25 µm film thickness) and a temperature program of 50° for 1 min, 16°/min to

80°, and then 7°/min to 200°C held 10 min. Subsamples (100 µl) from 10 to 12 of the cartridge extracts were pooled by species and sample type and concentrated approximately 10-fold passively on the lab bench. Antennae of male *D. frontalis* (5 antennal preparations) and *D. mesoamericanus* (6 antennal preparations) were tested with these concentrated volatiles extracts (2 µl into GC). A positive olfactory response was recorded if an EAD deflection was detected at a particular retention time in at least four GC-EAD runs. The identities of suspected olfactory stimulants (as identified by GC-MS analysis, see below) were in general confirmed by performing GC-EAD analyses with synthetic mixtures which included these suspect stimulant compounds purchased from commercial suppliers (Compounds without this additional step are identified in the results). Components in the synthetic mixtures were diluted 1/1000 by volume in hexane and only non-coeluting compounds were combined into the same GC-EAD test mixtures. For all experiments antennal preparations were used in merely a single GC-EAD analysis.

Quantification of Olfactory Stimulants. HID peaks coinciding with EAD responses were identified by analyzing the concentrated, pooled extracts by gas chromatography-mass spectrometry (GC-MS; Hewlett-Packard model G1800C, Palo Alto, California, United States of America) utilizing the same column, temperature program, and carrier flow rates as used in the GC-EAD analyses. Compound identifications were accomplished by matching both retention times and mass spectra to those of commercially-obtained compounds. The identified olfactory stimulants were then quantified in the individual (i.e., not pooled) extracts derived from single aerations of female frass. Quantifications were derived from single-ion abundance ratios between the target compound and the internal standard; these ratios were then converted to ng per sample with a calibration curve produced by analyzing serial dilutions of known quantities of the standard compounds.

Statistical Analyses. Olfactometer assays. Behavioral bioassay data for time spent within the response quadrant contained an excess of zeroes and could not be transformed to meet assumptions of normality. Therefore these data were analyzed by GLM with a negative binomial distribution (Lindén and Mäntyniemi 2011) with logarithmic link function for pairwise comparisons of means, and a Wilcoxon one sample test with a hypothetical median value equal of zero and a 95% confidence interval was applied to contrast between odorized and control quadrant when the latter had zero counts. The overdispersion was evaluated from deviance residuals values respecting degrees of freedom of the model. For all statistical tests $\alpha=0.05$. In the single-odor olfactometer bioassays, we tested whether males were attracted/arrested by an odor source by determining whether the time spent significantly exceeded the average time spent within the three control for each trial.

For odor choice tests, we established whether an odor preference existed and whether the beetles were responding to either odorized quadrant more than controls by contrasting the time spent in the response quadrant for each of the two odor-treated quadrant and the average time spent in the response quadrant of the two control quadrant.

For GC-EAD data, the amplitude of the male EAD voltage deflections in response to individual compounds present in volatiles from females of either species were contrasted within odor source (female frass) and species of male. Contrasts were made using a t-test when data were normally-distributed or a Kolmogorov-Smirnov test otherwise.

For statistical analysis of quantities of individual compounds detected in frass volatiles, compounds were first classified as either host volatiles or pheromones (Skillen et al. 1997), and then their quantities were statistically analyzed within these groupings. Data were normalized with a $3\sqrt{}$ (X+ 0.05) transformation and then analyzed by a one-way ANOVA.

Additionally, a t-test or Kolgomorov-Smirnov test were used to compare quantities of single compounds produced by females of either species. All statistical analyses were performed with SPSS Statistics Vol. 21.

RESULTS

Responses by Males to Volatiles from Female Frass in a Permeable Platform Olfactometer. In single-odor tests of male responses to female frass odors, both *D. frontalis* (Fig. 2A) and *D. mesoamericanus* (Fig. 2B) males spent significantly more time in the platform quadrant above the single, frass-filled ELISA plate well than the other quadrants (i.e., without frass-filled wells) when the frass was from conspecific females (for *D. frontalis* males: D=71.5, gl=142; $X^2_{0.05,\ 1}$=4.06, P=0.044; for *D. mesoamericanus* males: D=43.2, gl= 186; $X^2_{0.05,1}$=9.04, P= 0.003) but not heterospecific females (for *D. frontalis*: D=102, gl=142; $X^2_{0.05}$, 1=3.50, P= 0.061; for *D. mesoamericanus*: D=81.9, gl= 174; $X^2_{0.05}$, 1=3.43, P= 0.064). For males of both species, the time spent within the frass-containing quadrant was significantly greater when the frass was from conspecific females (for *D. frontalis*: D=70.0, gl=70; $X^2_{0.05,1}$=6.90, P=0.009; for *D. mesoamericanus*: D=89, gl= 89, $X^2_{0.05,1}$=6.64, P=0.001). In an odor choice test (i.e., when two frass-filled wells were available simultaneously in opposite quadrants, Fig. 3), males of both species spent significantly more time in the quadrant with frass of conspecific females than heterospecific females (for *D. frontalis*: D=62.7, gl=101; $X^2_{0.05,\ 2}$=37.3, P<0.001; for *D. mesoamericanus*: D=137, gl=161, $X^2_{0.05,2}$=13, P=0.002).

Electrophysiological Responses (GC-EAD). Electrophysiological recording was strongly consisting of EAG each test applied to the male antennae, so that the antennal activity of at least 15 compounds in frass produced by one or both females species were identified (Fig.

4). The mayor of compounds elicite EAD responses to both male species and only five of them (δ-3-carene, *endo*-brevicomin, limonene, nonanal and verbenone) correspond to only one male species. Only one response was related to one HID peak (peak 13; Fig. 4A, 4B) composed by coeluting 4-allylanisole and *trans*-verbenol (as well as ipsdienol in *D. mesoamericanus* samples). Coeluted compounds was confirmed by standards analyzed separately.

For males of both species, significant variation was observed in the amplitude of EAD responses to compounds collected from female frass of *D. frontalis* (for *D. frontalis* males: $F_{0.05,10}$=4.90, P<0.001; for *D. mesoamericanus* males: $F_{0.05,12}$=3.26, P=0.002, Fig. 4C, 4D) and *D. mesoamericanus* (for *D. frontalis* males: $F_{0.05,15}$=3.89, P<0.001; for *D. mesoamericanus* males: $F_{0.05,12}$=20.8, P<0.001, Fig. 4C, 4D). Frontalin, and *endo*-brevicomin elicited the strongest antennal responses in males of both species, whereas peak 13 EAD responses had differing intensities between males of either species where low and high amplitudes were registered when *D. frontalis* or *D. mesoamericanus* were tested, respectively (Fig. 4C, 4D).

Antennal response voltages produced by males sometimes differed at specific retention times in the EAD analyses depending on the species of female from which the test odors were derived. Such differing responses occurred at the retention time of *endo*-brevicomin for *D. frontalis* males responding to frass odors (Z=1.58, P=0.013); and for *D. mesoamericanus* males responding to frass odors (Z=1.65, P=0.009). In all cases, this response occurred with odors from *D. mesoamericanus* but not *D. frontalis* females. Also terpinen-4 ol elicited significative EAD responses of *D. mesoamericanus* males to frass odors from *D. frontalis* females ($t_{0.050,9}$= 2.3, P= 0.048). Since EAD response profiles by

males of both species were similar to data from EAD responses to frass volatiles of each female species were pooled by species of male and sample type to increase test power for contrasts of male EAD responses to odors of females of the two species. A t-test was then applied to contrast EAD response voltages produced at a specific retention time by odors of females of either species. In addition to the differences noted earlier, there were significant differences between male EAD responses to odors of females of either species to peak #13 ($t_{0.050,\ 19}$=-2.55, P=0.020).

Quantification of Olfactory Stimulants. Ten host compounds were identified from which six were monoterpenes (α-pinene, β-pinene, δ-3-carene, myrcene, limonene, and linalool), one sesquiterpene (longifolene), one terpene alcohol (terpinen-4-ol), one phenylpropanoid (4-allylanisole) and one aldehyde (nonanal), but only α-pinene, linalool and the sesquiterpene longifolene were the most abundant olfactory stimulants in odors collected from frass of either species, followed by host compounds 4-allylanisole, and the monoterpenes, myrcene, β-pinene, and limonene (Fig. 5A), but only δ-3-carene (t0.050, 12= -2.2, P= 0.049) and Linalool (t0.050, 12= -2.3, P= 0.041) differed significantly between odors associated with females of either species (Fig. 5A). Seven insect-produced compounds were identified by EAD sensitivity of which, two were bicyclic ketals (frontalin, endo-brevicomi), five were oxygenated monoterpene (*cis*-verbenol, *trans*-verbenol, verbenone, myrtenol, and ipsdienol) (Fig. 5B). Only two insect-produced compounds recovered from frass differed significantly in quantity between species, namely, *endo*-brevicomin (Z=1.63, P=0.010) and ipsdienol (Z=1.84, P=0.002) which were detected only from *D. mesoamericanus* females (Fig. 5B).

DISCUSSION

The evidence presented here confirms that semiochemicals produced by female *D. frontalis* and *D. mesoamericanus* play a significant role in male discrimination of females of either species (Niño et al., 2015). After arrivng at the host bole, males must find the gallery entrance of a solitary conspecific female, and close range pheromone signals apparently function in mate discrimination once the male is close to the source of pheromones, i.e., within millimeters of female frass. Since at least some frass remains outside of every single gallery entrance, frass clearly functions as a very important source of volatile signals used by males in recognizing the precise location of a female entrance. In the results, males of both *D. frontalis* and *D. mesoamericanus* responded much more strongly to volatiles from frass of conspecific females rather than heterospecifics. This occurred both when conspecific or heterospecific odors were presented as the only possible choice as well as when the male was presented with both simultaneously.

Thus for males of both species, discrimination of frass volatiles of the female species is at least in part mediated by female-produced semiochemicals and this is likely a very important function for frass, since every male crawling on the host bark is exposed to an odor plume composed of pheromones associated with single females, but also to pairs of both species too. In previous laboratory studies of *D. frontalis* and *D. mesoamericanus*, males confined over gallery entrances of heterospecific females often paired and mated (Armendáriz-Toledano et al. 2014), whereas gallery systems of syntopic populations in the field apparently rarely if ever contain heterospecific pairs (Niño et al. unpublished data). Since there is substantial attack overlap in space (i.e., intermixing of gallery entrances on the bark) and time (i.e., some simultaneous arrival and attack; authors' unpublished data) during host colonization by these species, important reproductive isolation mechanisms ostensibly act during the interval between male landing and pairing within a gallery. Our data suggest that

discrimination of female semiochemicals contained in the frass by males walking on the bark surface is a mechanism that could prevent or reduce heterospecific pairings by these two species.

Once mate-seeking males in the genus *Dendroctonus* locate the gallery entrance of a conspecific female or the frass being expelled from it, their movement is arrested (Ryker 1988; authors' observations). Evidence indicates that this arrestment is caused by the high concentration of female volatiles at the gallery entrance, (Rudinsky 1973; Rudinsky et al. 1974). Consistent with this, male movement in our study was arrested over wells filled with conspecific frass when these were covered with an odor-permeable platform (i.e. a screen), and we observed efforts by these males to dig through or otherwise penetrate the platform (watch video in supplementary material #1) as they naturally do in response to frass over a female gallery entrance.

Male antennae of either species were sensitive to a total of sixteen compounds associated with female frass, and any number of these could have played a role in mediating male behavioral responses. Terpenoids typically present in the defensive resin of pines (e.g., α-pinene, β-pinene, myrcene, and longifolene) were the predominant compounds in volatiles from frass, and presumably these originated from the damaged phloem tissue being mined and consumed by the beetles. The olfactory sensitivity by both species to these host-produced compounds may reflect their role in mediating host selection by members of the genus (Cano-Ramírez et al. 2012; Raffa 2001; Seybold et al. 2006). Pine monoterpenes can be potent attractants or attractive synergists of pheromones for pine-infesting bark beetles (Skillen et al. 1997) and the concentration-dependence of these responses may vary among species or guilds (Billings 1985; Erbilgin et al. 2003). The mean concentrations of

these host odors demonstrated a trend of being somewhat higher (although this was significant only for δ-3-carene and linalool) in samples from *D. mesoamericanus*, likely because this species produced frass in greater quantities than *D. frontalis* in our study. Differing overall release rates of resin-associated odors by attacks of the two species thus could conceivably play a role in species discrimination, but this possibility was not addressed by our study.

Some related species produce very similar volatiles profile, but differing responses to specific semiochemicals and their release rates could allow males to discriminate conspecific females. In our results using frass produced by females of each species as the source of semiochemicals, there were EAD responses of males of both species to at least fourteen volatiles present in frass produced by females of both species, but only for frass from *D. mesoamericanus* females did males display a response to *endo*-brevicomin and ipsdienol, because these two pheromones are produced in females only of this species. These pheromones have been proposed to mediate discrimination by walking males to females (Niño et al., 2015). In addition males of both species had an EAD-response to the minor host volatiles, δ-3-carene, linalool and nonanal, but the response corresponding to δ-3-carene was observed only with frass of *D. mesoamericanus*. However there was a significant difference in the concentrations for δ-3-carene and linalool associated with female frass of either species. Thus it is possible that these compounds play a role in mediating species discrimination, and possibly with greatest importance when close to a gallery entrance of a solitary female.

The results are consistent with earlier research (Niño et al. 2015) and confirm that males of *D. frontalis* and *D. mesoamericanus* recognize conspecific mates by discrimination of female

odors. They also confirm that between females of these two species there are differences in the volatiles profile, insofar as *endo*-brevicomin and ipsdienol production occurs only in *D. mesoamericanus* females and this represents a qualitative differences between females of either species (Niño et al. 2015). However, during host colonization both sexes contribute to the volatiles profile of a mass attack (Sullivan et al. 2012). In addition, bioassays employing a passive diffusion olfactometer (as were the tests of this paper), likely represent final odor recognition of a gallery entrance of a solitary, available female. In examining odors presumably involved in such close range mate discrimination, we were able to detect that male antenna of *D. frontalis* were sensitive to δ-3-carene which distinguishes frass volatiles of *D. mesoamericanus* females.

The significant differences of δ-3-carene concentration between frass volatiles from females of both species could merely be a coincidental aspect of beetle biology and not an adaptation for chemical ecology, that is, the greater body size of *D. mesoamericanus* could be related to greater frass production and thus also to a greater concentration of this and many of the other identified volatiles (except for nonanal, terpinen-4-ol, and verbenone, Fig. 5) as has been mentioned early. Thus in combination with results of earlier research (i.e., Niño et al., 2015), our examination of frass odors assumes that semiochemicals production combined with some aspects of the biology of both species, which might not have an obvious function on species interaction, could work in tandem to facilitate mate discrimination by males of both species. In contrast, volatiles of lower concentration (i.e., δ-3-carene) perceived at a very short distance could play an important role in mate recognition when males are in contact with the frass. However the effect of variation in volatiles concentration due to variable frass production (as well as possible male discrimination of δ-3-carene, limonene, nonanal and verbenone) were not investigated in this research.

However preliminary measurements (i.e., the average weight of frass from *D. frontalis* frass was 8.3 mg and from *D. mesoamericanus* frass was 24.1 mg, with n=4, t-test: $t_{0.050,\ 6}$= 0.003) suggests that a very interesting research topic could be developed regarding the role of frass quantity in species coexistence.

Chemical ecology in bark beetles probably involves one of the most complex communication systems in non-social insects, but it addresses only one aspect of bark beetle behavior and mate recognition. Research addressing other senses indicate that bark beetles use acoustic communication during interactions at the gallery entrance (Michael and Rudinsky 1972; Rudinsky and Michael 1973, Lindeman and Yack 2015). Also, it is probable that both *D. frontalis* and *D. mesoamericanus* use visual cues too, and that their combination with odor cues could play a very important role in mediating mate finding. It has been observed that pitch tubes of *D. mesoamericanus* are bigger than those of *D. frontalis*, and perhaps visual discrimination of these differing pitch tubes could be another cue used in discrimination, but this aspect wasn't tested in our study. Research regarding visual cues of frass, pitch tubes, and other aspects of the gallery entrance could help to complete our understanding of male mate-finding behaviors.

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REFERENCES

Anderbrant O, Schlyter F and Birgersson G. 1985. Intraspeciphic competition affecting parents and offspring in the bark beetle *Ips typographus*. Oikos 45: 89-98.

Armendáriz-Toledano F, Niño A, Sullivan BT, Macias-Samano J, Victor J, Clarke SR, Zuñiga G. 2014. Two species within *Dendroctonus frontalis* (Coleoptera: Curculionidae): evidence from morphological, karyological, molecular, and crossing studies. Ann Entomol Soc Am 107:11-27.

Armendáriz-Toledano F, Niño A, Sullivan BT, Kirkendall LR, Zúñiga G. 2015. A new species of bark beetle, *Dendroctonus mesoamericanus* sp. nov. (Curculionidae: Scolytinae) in southern Mexico and Central America. Ann Entomol Soc Am, published online.

Berryman AA, Dennis B, Raffa KF, and Stenseth NS. 1985. Evolution of optimal group attack, with particular reference to bark beetles (Coleoptera: Scolytidae). Ecology. 66:898-903.

Billings RF. 1985. Southern pine bark beetles and associated insects: Effects of rapidly-released host volatiles on response to aggregation pheromones. J Appl Entomol 99:483-491.

Birch MC. 1984 Aggregation in bark beetles. In: Bell WJ and Cardé RT (eds). Chemical ecology of insects. Chapman and Hall. Sinauer Assoc, Sunderland, Massachusetts, USA pp 331-353.

Blomquist GJ, Figueroa-Teran R, Aw M, Song M, Gorzalski A, Abbott NL, Chang E, Tittiger C. 2010. Pheromone production in bark beetles. Insect Biochemistry and Molecular Biology 40- 699-712.

Byers JA. 1989. Behavioral mechanisms involved in reducing competition in bark beetles. Holarct Ecol 12:466-476.

Byers JA and Zhang Q. 2011. Chemical ecology of bark beetles in regard to search and selection of host trees, pp. 132-150. In T. Liu and L. Kang (eds). Recent advances in entomological research. Higher Education Press, Beijing.

Cano-Ramirez C, Armendáriz -Toledano F, Macias-Samano JE, Sullivan BT, Zuñiga G. 2012. Electrophysiological and behavioral responses of the bark beetle Dendroctonus rhizophagus to volatiles from host pines and conspecifics. J Chem Ecol 38:512-534.

Denno RF, Mcclure MS, Ott JR. 1995. Interspecific interactions in phytophagous insects: competition reexamined and resurrected. Annu Rev Entomol 40:297-331.

Erbilgin N, Powell JS, Raffa KF. 2003 Effect of varying monoterpene concentrations on the response of Ips pini (Coleoptera: Scolytidae) to its aggregation pheromone: implications for pest management and ecology of bark beetles. Agric For Entomol 5:269-274.

Gries G, Pierce HD, Lindgren BS, and Borden JH. 1988. New techniques for capturing and analyzing semiochemials for Scolytid beetles (Coleoptera: Scolytidae). J. Econ. Entomol. 81: 1715-1720.

Hofstetter, RW, Gaylord ML, Martinson M, and Wagner MR. 2012. Attraction to monoterpenes and beetle-produced compounds by syntopic *Ips* and *Dendroctonus* bark beetles and their predators. Agricultural and Forest Entomology. 14: 207–215.

Lindeman AA and Yack JE. 2015. What is the password? Female bark beetles (Scolytinae) grant males access to their galleries based on courtship song. Behavioral Processes. 115: 123–131.

Lindén A and Mäntyniemi S. 2011. Using the negative binomial distribution to model overdispersion in ecological count data. J Ecol Soc Am 92: 1414-1421.

Michael RR and Rudinsky JA. 1972. Sound production in Scolytidae: specificity in male *Dendroctonus* beetles. J Insect Physiol 18:2189-2201.

Moreno CB. 2008. Diferenciación morfológica y comportamental de dos morfos de *Dendroctonus frontalis* Zimmermann (Coleoptera: Scolytinae) en Montebello, Chiapas. Thesis, El Colegio de la Frontera Sur, Chiapas, MX.

Moser JC, Fitzgibbon B and Klepzig KD. 2005. The Mexican pine beetle, *Dendroctonus mexicanus*: first record in the United States and co-occurrence with the Southern pine beetle, *Dendroctonus frontalis* (Coleoptera: Scolytidae or Curculionidae: Scolytinae). Entomol News 116:235-243.

Niño-Domínguez A, Sullivan BT, López-Urbina JH, and Macías-Sámano JE. 2015. Pheromones-mediated mate location and discrimination by two syntopic sibling species of *Dendroctonus* bark beetles in Chiapas, Mexico. J. Chem. Ecol. DOI 10.1007/s10886-015-0608-4.

Okland B, Skarpaas O, and Kausrud K. 2009. Threshold facilitations of interacting species. Popul Ecol 51:513-523.

Paine TD, Birch MC, Svihra P. 1981. Niche breadth and resource patitioning by four sympatric species of bark beetles (Coleoptera: Scolytidae). Oecologia 48:1-6.

Paine TD, Millar JG, and Hanlon CC. 1999. Identification of semiochemicals associated with jeffrey pine beetle *Dendroctonus jeffreyi*. J. Chem. Ecol. 25: 433-453.

Raffa KF. 2001. Mixed messages across multiple trophic levels: the ecology of bark beetle chemical communication systems. Chemoecology 11:49-65.

Renwick JAA and Vité JP. 1970. Systems of chemical communication in *Dendroctonus*. Contrib. Boyce Thompson Instit. 24: 283-292.

Rudinsky JA. 1973. Multiple functions of the southern pine beetle pheromone verbenone. Environ Entomol 2:511-514.

Rudinsky JA, Morgan ME, Libbey LM, and Putnam TB 1974. Antiaggregative-rivalry pheromone of the mountain pine beetle, and a new arrestant of the southern pine beetle. Environ Entomol 3:90-97.

Rudinsky JA and Michael RR. 1973. Sound production in Scolytidae: stridulation by female *Dendroctonus* beetles. J Insect Physiol 19:689-705.

Ryker LC. 1988. Acoustic studies of *Dendroctonus* bark beetles. Fla Entomol 71:447-471.

Schlyter F, Jakus R, Han F-Z, Ma J-H, Kalinová B, Mezei P, Sun J-H, Ujhelyiová L, and Zhang Q-H. 2015. Reproductive Isolation of *Ips nitidus* and *I. shangrila* in Montain Forest of Western China: Responses to Chiral and Achiral Candidate Pheromone Componentes. J. Chem. Ecol. DOI 10.1007/s10886-015-0594-6.

Seybold SJ, Huber DPW, Lee JC, Graves AD, and Bohlmann J. 2006. Pine monoterpenes and pine bark beetles: A marriage of convenience for defense and chemical communication. Phytochem Rev 5:143-178.

Six DL and Bracewell R. 2015. *Dendroctonus*. Pp. 305-350. In F. E. Vega and R. Hopsthetter R (eds). Bark Beetles Biology and Ecology of Native and Invasive Species. Elservier press, Oxford UK.

Skillen EL, Berisford CW, Camaan MA, and Reardon RC. 1997. Semiochemicals of forest and shade tree insects in North America and management applications. USDA Forest Service Forest Health Technology Enterprise Team Publication, FHTET 96-15.

Smadja C. y Butlin RK. 2009. On the scent of speciation: the chemosensory system and its role in premating isolation. Heredity. Vol. 102: 77–97.

Sullivan BT, Niño A, Moreno B, Brownie C, Macías-Sámano J, Clarke SR, Kirkendall LK, Zúñiga G. 2012. Biochemical evidence that *Dendroctonus frontalis* consists of two sibling species in Belize and Chiapas, Mexico. Ann Entomol Soc Am 105:817-831.

Sullivan BT. 2011 Southern pine beetle behavior and semiochemistry. In: Coulson RN, Klepzig KD (eds) Southern pine beetle II. USDA Forest Service Southern Research Station General Technical Report SRS-140, pp 25-50.

Rudinsky JA and Michael RR. 1973. Sound production in Scolytidae: stridulation by female *Dendroctonus* beetles. J Insect Physiol 19:689-705.

Vité JP and Pitman GB. 1968. Bark beetles aggregation: Effects of feeding on the release of pheromones *Dendroctonus* and *Ips*. Nature. 218: 169-170.

Wood DL. 1982. The role of pheromones, kairomones, an allomones in the host selection and colonization behavior of bark beetles. Ann Rev Entomol 27:411-446.

Zúñiga G, Mendoza CG, Cisneros R, y Salinas-Moreno Y.1999. Zonas de sobre posición en las aéreas de distribución geográfica de las especies mexicanas de *Dendroctonus* ERICHSON (Coleoptera: Scolytidae) y sus implicaciones ecológico-evolutivo. Acta Zool. Mexicana. No. 77: 1-22.

Figure Captions

Fig. 1 Layout of a permeable platform olfactometer constructed from a 96-well ELISA plate (10.8 x 14.6 cm) and used to test attraction of walking male beetles to odors of female frass. Wells indicated with broken lines (B4, B9, G9, and G4) were filled with female frass or left empty. In single odor assays a single, randomly-selected well of the four was filled; in odor choice assays, wells in a pair of opposite quadrants (B4-G9 or B9-G4, selected randomly) were filled. The surface of the plate was then covered by fine-mesh plastic screen and a test male released at the location indicated with a diamond. Time spent by the male in each of the four quadrants (5.4 X 7.3 cm) was recorded for 5 min following male release.

Fig. 2 Mean (±SE) time spent by walking male *D. frontalis* (A) and *D. mesoamericanus* (B) within quadrants dividing the arena of a screen-floored platform olfactometer. Female frass was placed into a plastic well underneath one of the four (5.4 x 7.3 cm) quadrants, and a male beetle was then released at the origin of the quadrants. The times spent by the males within the three quadrants without frass (controls) were averaged for statistical comparisons. An asterisk indicates that arrestment time within the frass-treated quadrant was significantly greater than the average time in the control quadrants; treatments labelled with different lower-case letters differed significantly in mean arrestment duration (GLM with negative binomial distribution and logit function, with $\alpha=0.05$).

Fig. 3 Mean (±SE) time spent by walking male *D. frontalis* and *D. mesoamericanus* within quadrants dividing the arena of a screen-floored platform olfactometer. Frass from females of either species was placed into single plastic wells beneath two opposite quadrants, and a

male beetle was then released at the origin of the quadrants. The times spent by the males within the two quadrants without frass (controls) were averaged for statistical comparisons. Within each species of male, treatments labelled with different lower-case letters differed significantly in mean arrestment duration (GLM with negative binomial distribution and logit function, with α=0.05).

Fig. 4 Coupled gas chromatography–electroantennographic detection (GC-EAD) analyses with the antennae of male *D. frontalis* **A**. and *D. mesoamericanus* **B**. responding to volatiles collected from gallery entrances occupied by a female of either species. The bar graph displays the average EAD response amplitudes (±SE) associated with individual helium ionization detector (HID) peaks in the GC-EAD runs as well as identifications of these HID peaks. Asterisks above error bars indicate that there was a significant difference in EAD response amplitude at the given retention time in response to females of either species (t-test or Kolgomorov-Smirnov test and α=0.050). Asterisks adjacent to a compound name indicate that olfactory sensitivity to the indicated compound was confirmed by means of GC-EAD analyses with commercially-obtained standards.

Fig. 5 Quantities (mean ± SE) of olfactory stimulants (i.e., compounds identified in GC-EAD studies illustrated in in Fig. 4) collected during dynamic headspace aerations of individual gallery entrances of solitary females. Compounds originating ostensibly from either the host tree tissue (A) or the beetle itself (B) are displayed separately. Quantities associated with the same lower case letters were not significantly different (one-way ANOVA, α=0.05). An asterisk indicates that the quantities of a particular compound produced by female entrances differed significantly between species (t-test with α=0.05).

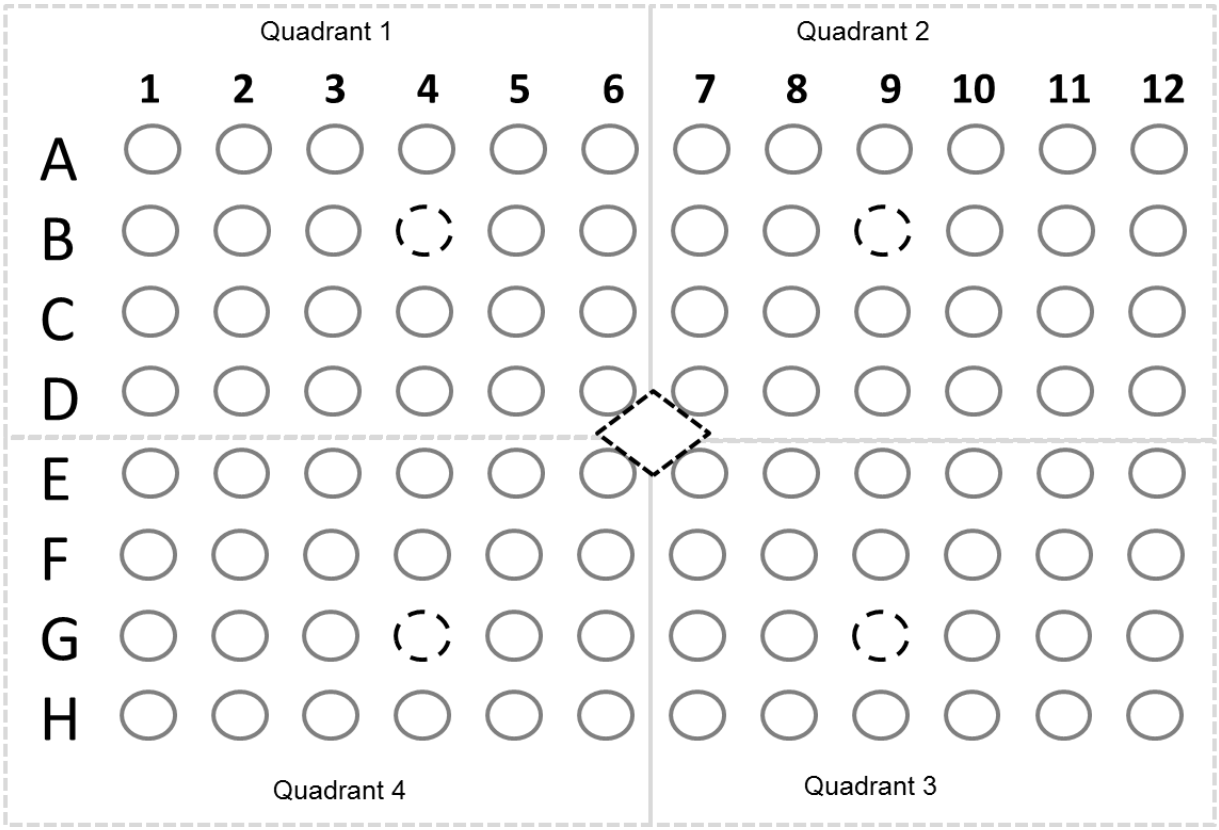
Quadrant 1
Quadrant 2
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
Quadrant 4
Quadrant 3

Figure 1.

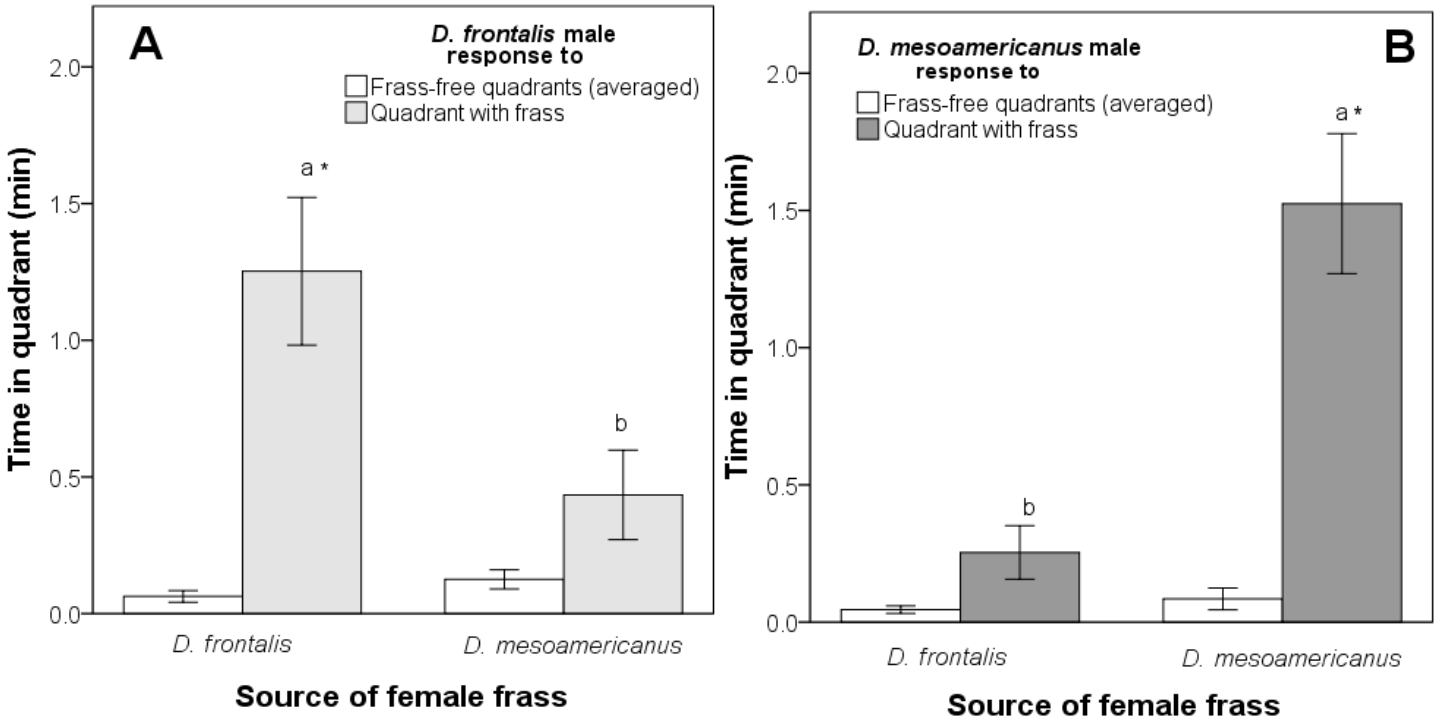

Figure 2.

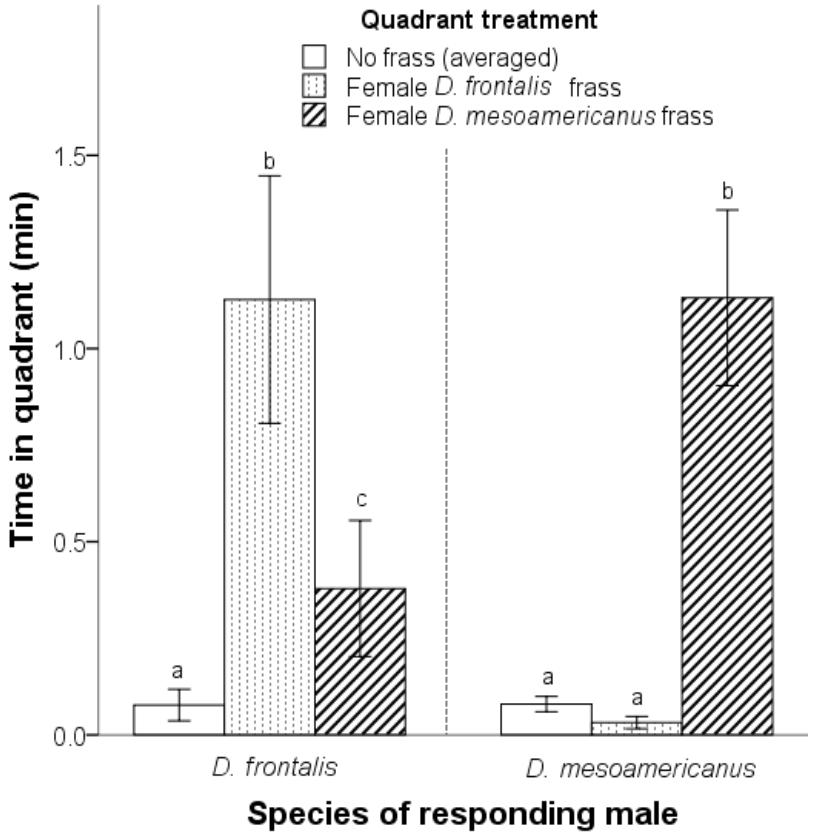

Figure 3.

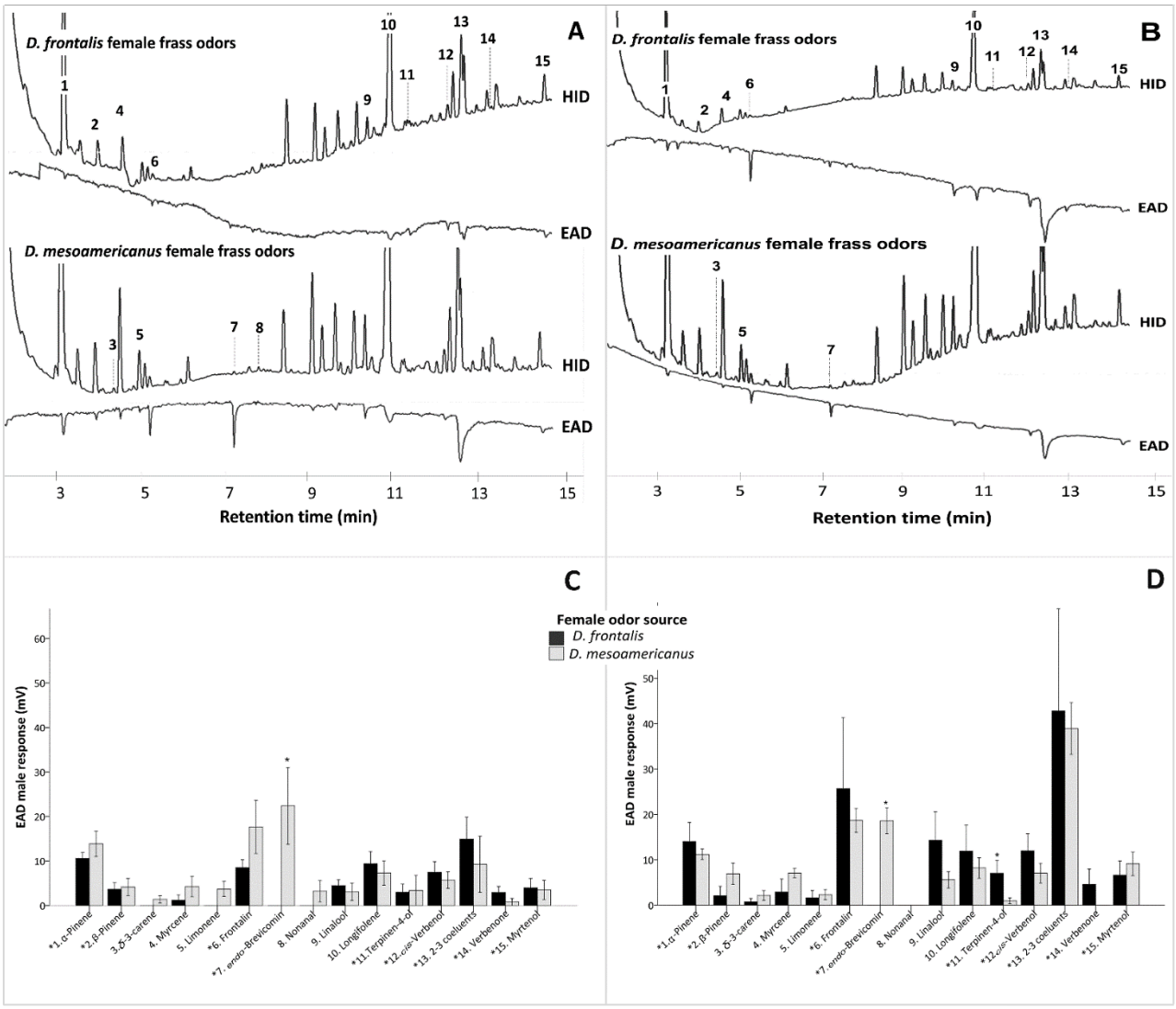
A
D. frontalis female frass odors
HID
EAD
D. mesoamericanus female frass odors
HID
EAD
Retention time (min)
B
D. frontalis female frass odors
HID
EAD
D. mesoamericanus female frass odors
HID
EAD
Retention time (min)
C
Female odor source
D. frontalis
D. mesoamericanus
EAD male response (mV)
*1. α-Pinene
*2. β-Pinene
3. δ-3-carene
4. Myrcene
5. Limonene
*6. Frontalin
*7. endo-Brevicomin
8. Nonanal
9. Linalool
10. Longifolene
*11. Terpinen-4-ol
*12. cis-Verbenol
*13. 2-3 coeluents
*14. Verbenone
*15. Myrtenol
D
EAD male response (mV)
*1. α-Pinene
*2. β-Pinene
3. δ-3-carene
4. Myrcene
5. Limonene
*6. Frontalin
*7. endo-Brevicomin
8. Nonanal
9. Linalool
10. Longifolene
*11. Terpinen-4-ol
*12. cis-Verbenol
*13. 2-3 coeluents
*14. Verbenone
*15. Myrtenol

Figure 4

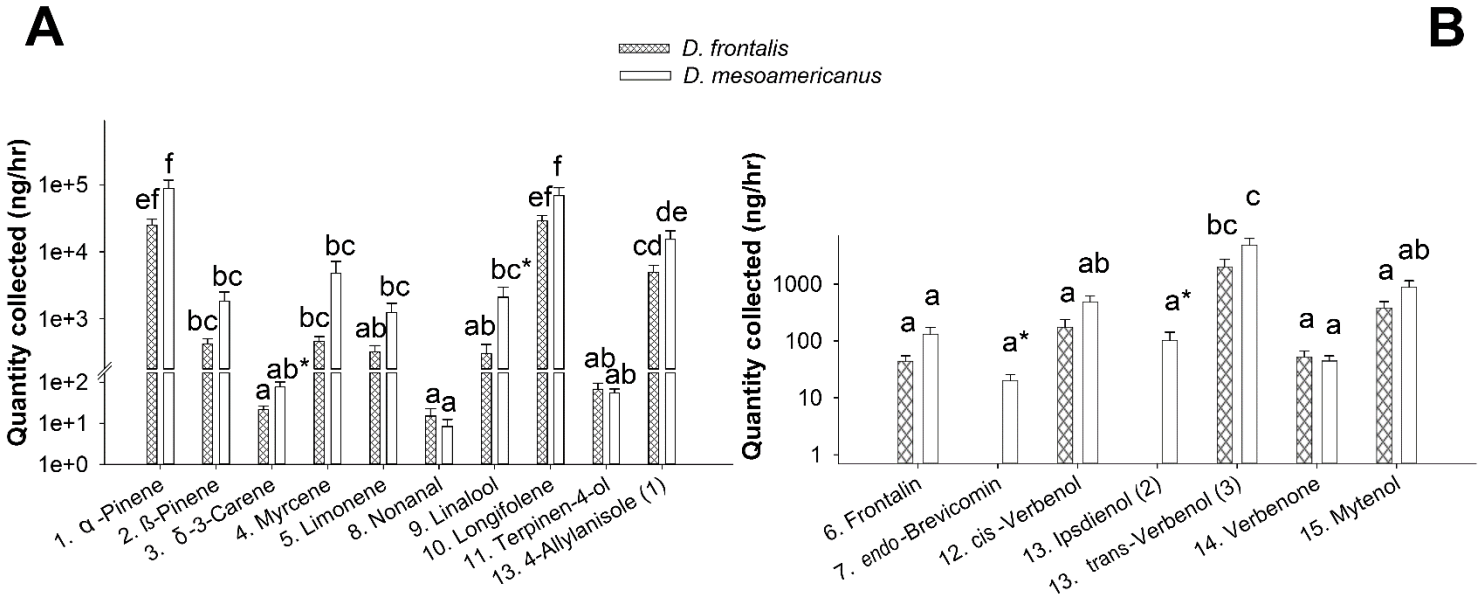
A
B
D. frontalis
D. mesoamericanus
Quantity collected (ng/hr)
1e+0
1e+1
1e+2
1e+3
1e+4
1e+5
1. α-Pinene
2. ß-Pinene
3. δ-3-Carene
4. Myrcene
5. Limonene
8. Nonanal
9. Linalool
10. Longifolene
11. Terpinen-4-ol
13. 4-Allylanisole (1)
Quantity collected (ng/hr)
1
10
100
1000
6. Frontalin
7. endo-Brevicomin
12. cis-Verbenol
13. Ipsdienol (2)
13. trans-Verbenol (3)
14. Verbenone
15. Mytenol

Figure 5.

CAPÍTULO IV

Responses by *Dendroctonus frontalis* and *D. mesoamericanus* (Coleoptera: Curculionidae) to semiochemical lures in Chiapas, Mexico: multiple roles of pheromones during joint host attacks

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Running Head:

Niño et al.: Semiochemicals of *D. frontalis* and *D. mesoamericanus*

Responses by *Dendroctonus frontalis* and *D. mesoamericanus* (Coleoptera: Curculionidae) to semiochemical lures in Chiapas, Mexico: multiple roles of pheromones during joint host attacks

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ABSTRACT

In southern Mexico and Central America, the southern pine beetle *Dendroctonus frontalis* Zimmermann (Coleoptera: Scolytinae) commonly colonizes host trees simultaneously with *Dendroctonus mesoamericanus* Armendariz-Toledano and Sullivan, a recently-described sibling species. We hypothesized that cross-species pheromone responses by host-seeking beetles might mediate joint mass attack, bole partitioning, and reproductive isolation between the species. Previous studies had indicated that *D. frontalis* females produce frontalin and that female *D. mesoamericanus* produce frontalin, *endo*-brevicomin, and ipsdienol (males of both species produce *endo*-brevicomin and possibly ipsdienol). In field trapping trials in the Mexican state of Chiapas, *Dendroctonus* frontalis was attracted to the lure combination of turpentine and racemic frontalin; racemic *endo*-brevicomin enhanced this response. In a single test, *D. mesoamericanus* was attracted to the combination of turpentine, racemic frontalin, and racemic *endo*-brevicomin after the addition of racemic ipsdienol; in contrast, racemic ipsdienol reduced responses of *D. frontalis*. Inhibition of *D. frontalis* was generated in both sexes by (+)- and racemic ipsdienol, but by (-)-ipsdienol only in females. Logs infested with *D. mesoamericanus* females (the pioneer sex in *Dendroctonus*) attracted both species in greater numbers than either *D. frontalis* female-infested or uninfested logs. Our data imply that *D. frontalis* may be more attracted to pioneer attacks of *D. mesoamericanus* females, and that this could be due to the presence of *endo*-brevicomin in the latter. Other possible intra- and interspecific functions of semiochemicals investigated in our experiments are discussed.

Key words Coexistence, new specie, *Dendroctonus mesoamericanus*, reproductive isolation mechanism, source partition.

INTRODUCTION

Semiochemicals play critical roles during colonization of new hosts by aggressive bark beetles (Coleoptera: Curculionidae: Scolytinae). These semiochemicals may include pheromones produced by con- and heterospecific beetles as well volatiles from the host tree (Wood 1982, (Borden 1974). Aggregation pheromones concentrate conspecific attacks on individual trees and thereby mediate mass attacks which overwhelm host defenses (Six and Bracewell 2015, Raffa 2001); anti-aggregation pheromones reduce attraction of conspecifics and regulate attack densities (Byers 1989). Pheromones may also function secondarily as kairomones or synomones and mediate interactions among multiple species attacking the same or adjacent hosts (Birch and Svihra 1979, Svihra et al. 1980, Birch et al. 1980, Schlyter and Anderbrant 1993, Ayres et al. 2001). Beetles may “eavesdrop” on aggregation pheromones of other species in order to locate their host resources, or through cross-attraction engage in joint mass attacks that mutually increase the probability of colonization success (Svihra et al. 1980, Wagner et al. 1985, Okland et al. 2009). Species-specific differences in pheromone composition, release timing, and spatial distribution of attacks along the host bole can reduce the potential deleterious effects of intermixing of species such as interspecific competition and hybridization (Byers and Zhang 2011).

The bark beetle *Dendroctonus frontalis* Zimmerman is an important mortality agent for *Pinus* spp. in the eastern and southwestern United States, Mexico, and Central America (Billings et al. 2004, Wood 1982). Across its wide distribution, *D. frontalis* is commonly found coexisting on the same hosts with other closely related species of bark beetles (Zúñiga et al. 1995, Moser et al. 2005, Davis and Hofstetter 2009). Within a zone extending from the state of Michoacan, Mexico to northern Nicaragua, *D. frontalis* commonly coexists on the same hosts with *D. mesoamericanus* Armendariz-Toledano and Sullivan, a recently described,

sibling species (Sullivan et al. 2012, Armendariz-Toledano et al. 2014, 2015). Like *D. frontalis*, *D. mesoamericanus* appears to be an aggressive, tree-killing bark beetle species capable of causing landscape-scale mortality of pines (Midtgaard and Thunes 2002, Armendariz-Toledano et al. 2015, author's observations).

Previous investigations indicated that both species utilize pheromones and that there are differences and overlap in pheromone composition which likely affect the species' interactions in nature. In reciprocal cross-attraction studies in an olfactometer, walking males of both species were more strongly attracted by odors arising from entrances of conspecific than heterospecific females (Niño-Dominguez et al. 2015). Volatiles collections and extracts of adult beetles indicated that females (the pioneer sex in the genus *Dendroctonus*) of both species produce the common *Dendroctonus* pheromone component frontalin, whereas female *D. mesoamericanus* additionally produce ipsdienol and *endo*-brevicomin (Sullivan et al. 2012). *endo*-Brevicomin is produced by males of both species and ipsdienol by at least some *D. frontalis* males. (Sullivan et al. 2012). In olfactometer studies, ipsdienol and *endo*-brevicomin strongly enhanced attraction of walking *D. mesoamericanus* males while inhibiting responses of *D. frontalis* males to frontalin and alpha-*pinene*, hence these two compounds likely mediate discrimination of female entrances by mate-seeking males and enhance reproductive isolation of the species (Niño et al. 2015).

Although olfactometer studies have characterized a sex pheromone for *D. mesoamericanus*, it is not clear whether these same compounds also function in attracting both sexes to host trees under attack (i.e., serve as an aggregation pheromone). Trap lures consisting of frontalin, *endo*-brevicomin and host odors (i.e., distilled pine resin) attract *D. mesoamericanus* in significant but very low numbers even in areas with substantial populations (Sullivan et al. 2012, authors' personal observations). Ipsdienol, which

significantly enhanced attraction of *D. mesoamericanus* males in some olfactometer trials, has not been included in experimental trapping lures (Niño et al. 2015). In contrast to *D. mesoamericanus*, flying *D. frontalis* of both sexes are attracted in large numbers to traps baited with the combination of frontalin, *endo*-brevicomin, and host odors (distilled pine resin or purified *alpha*-pinene), which appears to function as their aggregation attractant (Sullivan 2011, Moreno et al. 2008). Since flying beetles of both species respond behaviorally to at least two pheromone components produced by the other species (i.e., frontalin and *endo*-brevicomin), these data imply that semiochemicals should influence interspecific interactions during joint host colonization. In particular, these data suggest that some cross attraction likely occurs. However, it is unlikely that these synthetic lures are fully representative of the odors produced by naturally-occurring attacks, and trials with natural (and thus presumably complete) sources of semiochemicals are needed.

The objective of this study was to determine whether odors previously identified in laboratory olfactometer assays as affecting inter- and intraspecific responses of *D. frontalis* and *D. mesoamericanus* produce similar effects on flying insects. In the aforementioned olfactometer tests, both the natural odor blend produced by female attacks of either species (air from female gallery entrances) and two compounds that qualitatively distinguished these odors (*endo*-brevicomin and ipsdienol) elicited strong discrimination by male beetles of both species (Sullivan et al. 2012, Niño et al. 2015). We therefore hypothesized that flying beetles should likewise demonstrate a strong preference for logs infested with female conspecifics, and that the components of the attractive olfactometer blends (including some or all of frontalin, *endo*-brevicomin, ipsdienol, and the host odor alpha-pinene) might be combined to produce attractive and species-specific trapping lures. Information on cross-attraction and cross-inhibition of odor blends should elucidate the role of specific

semiochemicals in attack synchronization, host partitioning, and reproductive isolation between these two species during co-colonization of hosts.

METHODS AND MATERIALS

Trapping experiments were conducted in a mixed pine-oak forest (with the predominant pines being *Pinus oocarpa* Shiede and *P. maximinoi* H.E. Moore) within Lagunas de Montebello National Park, La Trinitaria, Chiapas, Mexico (16° 07'1.93" N, 91° 44' 8.57" W). The area was experiencing a low-level outbreak of *D. frontalis* and *D. mesoamericanus* in which individual infested trees and flying beetles were present year-round and localized infestations occurred during the fall months.

Trapping experiments with synthetic semiochemicals. Twelve-unit multiple funnel traps (Synergy Semiochemicals, Burnaby, BC, Canada) were suspended from metal poles with the trap bottoms positioned at least 50 cm above of ground. Trap cups contained either a piece of fumigant insecticide strip (VAPORTAPE, Ercon Environmental, Pennsylvania, United States of America) or a 10:1 solution of water and liquid soap to retain and kill captured insects. Unless noted otherwise, traps were placed >100 m from the nearest *Dendroctonus* spp. infested tree, and >20 m from other pines. Lines of traps composing statistical blocks were >500 m apart whereas traps within these blocks were 100-200 m apart. Except for the turpentine releasers (which were suspended inside the top funnel) lures were attached midway between top and bottom of each trap. Insects collected from trap cups were preserved in 70% alcohol, and catches of *D. frontalis* and *D. mesoamericanus* were distinguished by the presence of diagnostic striations on the preepisternal area of the prothorax and a reduced mycangium on females of the latter species; beetles were sexed by the prominence of the frontal tubercles in males

(Armendáriz-Toledano et al. 2015). The origin, enantiomeric composition, release rate, and purity of lure contents and devices are given in Table 1.

Experiment 1. We compared beetle responses to six different lure combinations of racemic pheromone components and host odors (in the form of turpentine): (1) turpentine + frontalin, (2) turpentine + ipsdienol, (3) turpentine + *endo*-brevicomin, (4) turpentine + frontalin + ipsdienol, (5) turpentine + frontalin + *endo*-brevicomin, and (6) turpentine + frontalin + *endo*-brevicomin + ipsdienol. Three randomized complete blocks (with each block consisting of a line of six traps with one of the six treatments assigned randomly to each) were deployed. Accumulated catches were collected every ten days at which time treatment positions were re-randomized without replacement within blocks. The experiment was completed in 51 days (lure positions were re-randomized twice). The experiment was executed 7 April to 28 May 2013.

Experiment 2. The six lure treatments of experiment 1 were re-tested with an additional three: 7) turpentine alone, 8) turpentine + *endo*-brevicomin + ipsdienol, and 9) frontalin + *endo*-brevicomin + ipsdienol. These treatments were included to ascertain the separate importance of frontalin and turpentine in producing the observed attraction by *D. mesoamericanus* in experiment 1 to the four-component blend, and allow comparisons of pheromone-containing lure combinations to those of host odors alone. Three randomized complete blocks (with each block consisting of a line of nine traps with one of the nine treatments assigned randomly to each) were established with >100 m between blocks and >100 m between traps within blocks. Catches were collected daily at which time lure positions were re-randomized without replacement within each block. Nine randomizations/collections were made during the experiment such that every lure treatment was at every trap position for a single day/collection. This procedure resulted in three 9x9

Latin squares with traps within each block as columns and collection dates as rows. The experiment was conducted 13-21 November 2013.

Experiment 3. This 2-part test was performed to determine the possible effects of the enantiomeric composition of *endo*-brevicomin and ipsdienol on responses to the tested lures. In experiments 1 and 2, lures of these two compounds were racemic, whereas the enantiomeric compositions produced by female *D. mesoamericanus* are >99% (+) and >95% (+), respectively (Niño-Dominguez et al. 2015). Since antipodes of *endo*-brevicomin and ipsdienol respectively have been demonstrated to sometimes have antagonistic effects with *Dendroctonus* or *Ips* bark beetles (Vité et al. 1985, Seybold 1993), we wished to see if such antagonism might have affected the outcome of experiments 1 and 2. Frontalin enantiomers have not been shown to be antagonistic in *Dendroctonus* including *D. frontalis* (Wood et al. 1976, Payne et al. 1982, Lindgren 1992). In experiment **3A** the tested lure combinations included turpentine, racemic frontalin, racemic *endo*-brevicomin and (1) no additional compound, (2) racemic ipsdienol, (3) (+)-ipsdienol, or (4) (-)-ipsdienol. Three randomized complete blocks of four traps each were established, and the experiment was executed as a multiple Latin-square design similar to experiment 2. Insect collections and lure re-randomizations were performed daily on 1-5 November 2013. In experiment **3B** the tested lure combinations included turpentine, frontalin, and (1) racemic *endo*-brevicomin + racemic ipsdienol, (2) (+)-*endo*-brevicomin + racemic ipsdienol, and (3) racemic *endo*-brevicomin + (+)-ipsdienol. Three randomized complete blocks of three traps each were established, and the experiment was executed as a multiple Latin-square design similar to experiment 2. Insects were collected and treatment positions re-randomized daily for three days. The experiment was completed 19-21 of November 2014. (+)-*endo*-Brevicomin was released from a glass capillary (1 mm i.d.) which had the bottom end heat-sealed and was

filled to 1 cm below the capillary opening. The capillary was secured open-end-up inside of an open, inverted 4 ml capacity glass vial (Sullivan and Mori 2009) that was taped to a trap spoke.

Trapping experiments using natural semiochemical sources (Experiment 4). We tested beetle responses to logs (30 cm long; 12-15 cm diam.) of healthy *P. oocarpa* that were infested with 30 females of either species or left uninfested (three treatments). Females had emerged during the previous 3 d from naturally-infested logs collected at the park. Beetles were collected daily as they emerged within cloth bag enclosures, and collected beetles were held in refrigeration on moistened paper wipers prior to use. To force attacks, beetles were confined by screen disks within evenly-distributed pits drilled into the bark (Niño et al. 2015); uninfested control logs received pits but no beetles. After 12-15 h, the screen disks were removed, and the bolts were enclosed in zippered, fine nylon-mesh screen bags to prevent unintended beetle attacks when in the field.

In the field, logs were suspended from pipe standards between two 12-unit multiple funnel traps. Additionally, two black plastic cards (11 cm x 14 cm) coated with insecticide-impregnated adhesive were also attached on opposite sides of each screen bag at 90° from the funnel traps. Both sticky cards and funnel traps were employed in order to help insure sufficient captures. Three randomized complete blocks of three log-traps each were established, and the experiment was executed as a multiple Latin-square design similar to experiment 2. Blocks (and traps within blocks) were arranged as segments of a ring surrounding a small *Dendroctonus* infestation; traps were located between 20-150 m from the nearest infested tree. This intentional placement of traps in relatively closer proximity to infested trees was done to help insure sufficient catches of beetles for statistical analysis. Traps were spaced 25-35 m apart within blocks and >25 m between blocks. Trapped insects

were collected (and sticky cards replaced with new) and log treatments were re-randomized daily for three days. Captures by the cards and funnel traps were summed for analyses. Within 12 h of the completion of trapping, the infested logs were dissected to determine the numbers of females still present in each treatment.

Statistical Analysis. All insect catches consisted of non-normally distributed data, with many treatments having zero catches. For all analyses of data sets that included zero-catching treatments we used an X+1 transformation, then a generalized linear model (GLM) analysis. For experiments 1 and 2, we applied a GLM analysis with blocks and treatment as factors and for experiment 3A, 3B, and 4, a GLM analysis with rows, columns and treatment as factors. Effects of treatment, sex, the interaction between sex and treatment, and the effect of treatment within sex were tested for significance. A Poisson or negative binomial distribution was applied to all data according to best obtained fit. For experiments 1, 3A and 4 we used a negative binomial distribution and for experiment 2 and 3B a Poisson distribution. Both analyses employed a logarithmic function with $\alpha = 0.05$; following of treatment pairwise contrast within sex or treatment when sex response was mixed. A Mann-Whitney test was used for pairwise-contrasts for sex within each treatment, including comparisons when one treatment of the contrast caught no insects.

RESULTS

In experiment 1, *D. frontalis* catches were influenced by treatment ($D=398.3$, $gl= 678$, $X^2_{0.050,\ 5}= 44.6$, $P< 0.001$), sex ($D=189.5$, $gl= 682$, $X^2_{0.050,\ 1}= 5.3$, $P= 0.022$) and a treatment by sex interaction ($D=420.5$, $gl= 672$, $X^2_{0.050,\ 11}= 539.9$, $P< 0.001$). Responses by each sex of *D. frontalis* to traps were significantly affected by lure treatment (for males: $D=224.7$, $gl= 336$, $X^2_{0.050,\ 5}= 72.8$, $P< 0.001$; for females: $D=193.9$, $gl=336$, $X^2_{0.050,\ 5}=38.1$, $P<0.001$). For both

sexes the most attractive lure was the turpentine/frontalin/*endo*-brevicomin combination (Fig. 1). Elimination of either frontalin or *endo*-brevicomin significantly reduced catches of this three-component lure. Addition of ipsdienol to either the turpentine/frontalin or the turpentine/frontalin/*endo*-brevicomin combinations significantly reduced attraction. Males and females were caught in significantly different numbers by lure treatments turpentine/frontalin (Z=-3.73, P<0.001), turpentine/frontalin/ipsdienol (Z=-2.26, P=0.024), and turpentine/frontalin/*endo*-brevicomin/ipsdienol (Z=-3.32, P=0.001); for all of these contrasts more males than females were trapped. For *D. mesoamericanus* (which were caught in very low numbers; total catch = 16) there was a significant effect just to treatment (D= 439.8, gl= 678, $X^2_{0.050,\ 5}$= 34.2, P< 0.001). With sexes combined, there was a significant treatment effect for lure blend (D=194.8; gl= 336; $X^2_{0.050,\ 5}$= 27.5; P<0.001). *Dendroctonus mesoamericanus* was attracted to the complete mix of turpentine/frontalin/*endo*-brevicomin/ipsdienol in greater numbers than any other tested combination of components (Fig. 2). Elimination of *endo*-brevicomin and/or ipsdienol from this four-component blend significantly reduced beetle attraction. The two lure combinations lacking frontalin caught no *D. mesoamericanus*.

In experiment 2, there were significant effects for treatment (D=48.0, gl= 461; $X^2_{0.050,\ 8}$=76.1, P<0.001), sex (D=116.0, gl= 468; $X^2_{0.050,\ 1}$=8.1, P=0.004), and a treatment by sex interaction (D=39.1, gl= 452; $X^2_{0.050,\ 17}$=85.0, P<0.001) for *D. frontalis*. Both female (D=20.3, gl=38, $X^2_{0.050,\ 8}$=33.3, P<0.001) and male (D=107.5, gl= 218, $X^2_{0.050,\ 8}$=112.7, P<0.001) *D. frontalis* differed significantly in response to different lure blends, and, as in experiment 1, the blend turpentine/frontalin/*endo*-brevicomin was more attractive to either sex of *D. frontalis* than the other combinations (Table 1). Elimination of either frontalin or *endo*-brevicomin, or the addition of ipsdienol, significantly reduced attraction of either sex to this three component

mixture. Turpentine by itself was unattractive to *D. frontalis*, whereas elimination of this host kairomone from the four component lure (turpentine/frontalin/*endo*-brevicomin/ipsdienol) significantly reduced catches of females. Significant differences in male and female catches were detected for the turpentine/frontalin/*endo*-brevicomin (Z=2.536, P=0.011) and turpentine/frontalin treatments (Z=-2.575, P=0.010) to which significantly more males than females responded. For *D. mesoamericanus* there was not a significant treatment, sex, or treatment/sex interaction effect (Table 1). However catches of *D. mesoamericanus* were extremely low (two or fewer beetles were trapped by any lure treatment).

In experiment 3A, there was a significant effect for treatment (D= 23.6, gl= 86, $X^2_{0.050,\ 3}$=12.6, P=0.005), sex (D= 30.8, gl= 88, $X^2_{0.050,\ 1}$=5.4, P=0.020) and a treatment by sex interaction (D= 18.3, gl= 82, $X^2_{0.050,\ 7}$=17.9, P=0.012) where both sexes of *D. frontalis* (for females: D= 20.323, gl= 38, $X^2_{0.050,\ 3}$=16.749, P=0.001; for males: D= 11.428, gl=38, $X^2_{0.050,\ 3}$=23.982, P<0.001), showed significant preferences for treatment blends. Both racemic and (+)-ipsdienol reduced the attraction of both sexes of *D. frontalis* to the blend of turpentine, frontalin, and *endo*-brevicomin, whereas (-)-ipsdienol had no effect on male catches but reduced catches of females (Table 2). The Mann-Whitney U test did not detect differences between male and female catches within any treatment. For *D. mesoamericanus* catches, there were no significant effects for treatment or sex, but there was a significant treatment by sex interaction (D=64.9, gl=82; $X^2_{0.050,\ 7}$=14.2, P= 0.042), and male catches demonstrated a significant treatment effect (D=35.6, gl=38; $X^2_{0.050,\ 3}$=10.310, P= 0.016). For males, the blend of turpentine, frontalin, *endo*-brevicomin, and ipsdienol was more attractive when the ipsdienol was the (-)-enantiomer (trapping 5 insects) rather than the (+)-enantiomer or the racemate (which caught no beetles).

In experiment 3b, there was a significant effect of sex for *D. frontalis* captures (D= 39.6, gl= 48; $X^2_{0.050,\ 1}$= 4.6, P=0.037; with more males being trapped), but no effect of lure treatment, nor a treatment by sex interaction. For *D. mesoamericanus,* there was no effect due to treatment, sex, or a treatment by sex interaction. However, catches were extremely low for both sexes (only 1-2 insects per treatment) (Table 2).

In experiment 4, both species discriminated among log treatments used as trap lures. For *D. frontalis* (Fig. 3) there was a significant effect for log treatment (D=13.1; gl= 47; $X^2_{0.050,\ 2}$= 6.49, P= 0.039) but not a sex or a sex by treatment interaction. With sexes summed, there was likewise a significant effect for log treatment (D=10.5; gl= 20; $X^2_{0.050,\ 2}$= 9.34, P= 0.009), with higher numbers of *D. frontalis* trapped at logs infested with *D. mesoamericanus* females than at either *D. frontalis* female-infested logs or uninfested logs ($X^2_{0.050,\ 2}$= 14.2, P= 0.001), and these latter two treatments did not differ significantly in their catches. *Dendroctonus mesoamericanus* catches (Fig. 4) were affected by treatment (D= 44.8, gl= 47, $X^2_{0.050,\ 2}$= 40.2, P< 0.001), sex (D= 37.6, gl= 48, $X^2_{0.050,\ 1}$= 8.65, P= 0.003), and a treatment by sex interaction (D= 2.34, gl= 44, $X^2_{0.050,\ 5}$= 70.3, P< 0.001). Females were trapped in very low numbers and there was not a significant effect for log treatment (D=15.6, gl=20, $X^2_{0.050,\ 2}$= 1.84, P=0.398). However males strongly discriminated among log treatments (D= 22.9, gl= 20, $X^2_{0.050,\ 2}$= 25.9, P<0.001) with higher catches at conspecific female-infested logs than at either *D. frontalis* female-infested or uninfested logs (which caught no *D. mesoamericanus*). Significantly more male than female *D. mesoamericanus* were trapped at logs infested with conspecific females (Z=-2.800, P= 0.011). Within 12 h of completion of the experiment, 23-27 females were still present within nuptial chambers of the bait logs for either species.

DISCUSSION

Our study detected differences in the response of flying *D. frontalis* and *D. mesoamericanus* to synthetic or natural pheromone sources, and these results are consistent with the hypothesis that the two species have differing colonization strategies when they are co-infesting trees. *Dendroctonus mesoamericanus* was previously shown to be attracted to the lure combination of (±)-frontalin, (±)-*endo*-brevicomin, and turpentine (Moreno 2008), and our data suggest that (±)-ipsdienol may further enhance this combination as a trap lure for this species. This effect was detected in only one test (experiment 1) which had very low catches.The effect was not observed in experiments 2 and 3a which included this same treatment contrast but trapped even fewer insects and were not statistically significant for lure treatment. Similar attractive responses were observed in laboratory assays in which attraction of walking male *D. mesoamericanus* was enhanced when (±)-ipsdienol was added to this same three component blend of frontalin, *endo*-brevicomin, and host odors (i.e., racemic *alpha*-pinene) (Niño et al. 2015).

For *D. frontalis* the results of experiments 1 and 2 resemble those of previous studies that demonstrated synergism among frontalin, *endo*-brevicomin, and host odors as trap lures for both sexes of *D. frontalis* in the southeastern United States (Vité et al. 1985, Sullivan et al. 2007) and in Chiapas, Mexico (Moreno et al. 2008). Additionally, both racemic and (+)-ipsdienol substantially reduced responses of one or both sexes of *D. frontalis* to attractive blends (i.e., turpentine and frontalin, either with or without *endo*-brevicomin; experiments 1, 2, and 3a) whereas the (-)-enantiomer reduced responses of females but not males (experiment 3a). Ipsdienol has been found in paired male *D. frontalis* in Chiapas (Sullivan et al. 2012) and Arizona (author's unpublished data), but it has not been reported from populations in the eastern United States (Sullivan 2011). Our study represents the first evidence that ipsdienol is an attraction inhibitor for *D. frontalis* and, since it can be produced

by *D. frontalis* that it could possibly function as an antiaggregation pheromone component for this species. Ipsdienol has previously been identified in just two additional species of *Dendroctonus* (*D. brevicomis* LeConte and *D. ponderosae* Hopkins) and, as with *D. frontalis*, only in males and predominantly as the (+)-enantiomer (Byers 1982, Hunt et al. 1986). For both species ipsdienol inhibits response to attractant baited traps, and it may likewise be an antiaggregation pheromone for them (Byers 1982, Hunt and Borden 1988). *Dendroctonus mesoamericanus* thus appears to be unique in that ipsdienol is produced by both sexes (Sullivan et al. 2012) and may enhance rather than reduce attraction of conspecifics. The inhibition of attraction by ipsdienol may additionally mediate *D. frontalis* avoidance of pines being attacked by potentially competing species of *Ips* that produce ipsdienol (Hofstetter et al. 2012). However, our data suggest that avoidance would be greater for *Ips* spp. that produce relatively greater quantities of the (+)-enantiomer.

Since ipsdienol appears to have opposite effects on flying individuals of either species, this semiochemical could promote spatial separation of attacks and landings by the two species and thereby play some role in mediating partitioning of the host resource between them. Also, since ipsdienol is produced by female *D. mesoamericanus* but not female *D. frontalis*, it could mediate avoidance by flying males of portions of the bole with solitary females of the other species. Laboratory olfactometer assays indicated that ipsdienol (in addition to *endo*-brevicomin) likely plays a parallel role for males after landing on the host in allowing them to discriminate gallery entrances of con- and heterospecific females. Thus the interspecific difference in response to ipsdienol by both flying and walking males could presumably enhance reproductive isolation (Niño et al. 2015).

The aggregation pheromone of *D. frontalis* includes both frontalin and (+)-*endo*-brevicomin, with the latter being contributed by males after they land and join a female (Vité et al. 1985,

Sullivan et al. 2007). Consequently attacks by *D. frontalis* pairs can be substantially more attractive to conspecifics than attacks by solitary females (Sullivan et al. 2007, but see Svihra 1982). Thus the mechanism for the preference of flying *D. frontalis* for odors of female *D. mesoamericanus* attacks in logs is likely that female *D. mesoamericanus* produce both of these components of the aggregation pheromone for *D. frontalis* [i.e., frontalin and (+)-*endo*-brevicomin; Sullivan et al. 2012, Niño et al. 2015] whereas female *D. frontalis* produce only frontalin. This inference is supported by the equal numbers of male and female *D. frontalis* that were attracted to logs with female *D. mesoamericanus*; in contrast, responding *D. mesoamericanus* were almost entirely males. Thus it is likely that this preference for heterospecifics by *D. frontalis* might apply only to attacks by solitary females of *D. mesoamericanus* and not those of beetle pairs, and future experiments should investigate this. The apparent preference by flying *D. frontalis* males for odors of female *D. mesoamericanus* over female conspecifics is the reverse of what was observed in a walking olfactometer study in which odors of conspecific female attacks were strongly preferred (Niño et al. 2015). Again, this contrast was likely due in part to the production of *endo*-brevicomin by *D. mesoamericanus* females. *endo*-Brevicomin can disrupt attractive responses by walking male *D. frontalis* over a range of doses (Rudinsky et al. 1974, Niño et al. 2015), despite its capacity to be a potent attractive synergist for flying *D. frontalis* (Sullivan 2011). The preference of male *D. frontalis* while walking but not in flight for odors of unpaired conspecific females suggests that semiochemical-mediated location of potential mates by male *D. frontalis* occurs predominantly following landing on the host. Prior to landing, a positive response to *endo*-brevicomin likely functions in bringing male *D. frontalis* to a host tree undergoing mass attack by either one or both species and thus to a location where mates and food likely are present.

In contrast to *D. frontalis*, flying male *D. mesoamericanus* showed no cross-attraction to traps baited with *D. frontalis* female-infested logs, and, likewise, *D. mesoamericanus* were not attracted to traps baited with synthetic odor blends associated with *D. frontalis* females (i.e., frontalin with host odors alone; Sullivan et al. 2012; present study). This preference shown by flying *D. mesoamericanus* resembled the strong preference of walking male *D. mesoamericanus* for odors of conspecific female entrances in olfactometer studies (Niño et al. 2015). In these olfactometer studies, *D. mesoamericanus* displayed minimal attraction to odors of *D. frontalis* females, and this was apparently due to the absence of ipdienol and *endo*-brevicomin in association with entrances of *D. frontalis* females. Responses of *D. mesaomericanus* males to logs infested with conspecific females but not female *D. frontalis* suggests that both host- and mate-recognition for these males occurs through the same set of semiochemical cues.

In the genus *Dendroctonus,* females are the sex that selects new hosts and initiates mass attacks through their release of all or part of the aggregation pheromone. The results of the trap-log study imply that both *D. frontalis* and *D. mesoamericanus* should be more attracted to such “pioneer” attacks by *D. mesoamericanus* females than those of *D. frontalis* females. This would suggest that pioneer attacks by female *D. mesoamericanus* might possess a significantly greater likelihood of success in initiating mass attacks by one or both species and accelerate colonization. In trees that are jointly colonized, however, it is not yet known if one species predominantly initiates the mass-attack.

Paired male *D. frontalis* sampled in Arizona and Chiapas, Mexico produce *ipsdienol* along with *endo*-brevicomin (Sullivan et al. 2012, authors’ unpublished data), which appears to have opposing effects on *D. frontalis* response to frontalin/host odor baited traps. Simultaneous production of pheromone components with apparently opposing behavioral

effects is not uncommon in *Dendroctonus* (Conn et al. 1983, Byers et al. 1984, Pureswaran et al. 2000), and differing dose-thresholds could presumably determine which pheromone component's activity dominates during intraspecific and interspecific signaling. The current study examined only a single release rate of ipsdienol and frontalin, and rates we used were determined by the characteristics of the commercial lures and not the proportions or release rates of the naturally-produced enantiomers.

Our study utilized racemic frontalin and *endo*-brevicomin in all tests except in experiment 3b [which attempted to compare beetle response of (+)-*endo*-brevicomin and the racemate], however catches were too low to be informative. This test was done to determine whether the use of the racemic rather than solely the (+)-enantiomer (as produced by females of both species) might be one possible reason for low attraction of *D. mesoamericanus* to synthetic lures. Although the enantiomeric ratios of both frontalin and *endo*-brevicomin produced by *D. frontalis* depart strongly from racemic [≥85% (-) frontalin and >99% (+)-*endo*-brevicomin (Payne et al. 1982, Sullivan et al. 2007, Niño et al. 2015)], published data suggest that behavioral responses of this species differ little if at all when either the racemic mixture or the insect-produced enantiomeric ratio are used in trap lures (Payne et al. 1982, Vité et al. 1985, Sullivan and Mori 2009, Sullivan et al. 2011). Our results imply that the presence or absence of racemic or (+)-ipsdienol in trapping lures would provide some capacity to target either *D. mesoamericanus* and *D. frontalis,* respectively.

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REFERENCES

Armendariz-Toledano, F., A. Niño, B. T. Sullivan, J. Macias-Samano, J. Victor, S. R. Clarke, and G. Zuñiga.2014. Two species within *Dendroctonus frontalis* (Coleoptera: Curculionidae): evidence from morphological, karyological, molecular, and crossing studies. Ann. Entomol. Soc. Am. 107:11-27.

Armendáriz-Toledano, F., A. Niño-Dominguez, B. T. Sullivan, L. R. Kirkendall, and G. Zúñiga. 2015. A new species of bark beetle, *Dendroctonus mesoamericanus* sp. nov. (Curculionidae: Scolytinae) in southern Mexico and Central America. Ann. Entomol. Soc. Am 1-12.

Ayres, B. D., M. P. Ayres, M. D. Abahamson, S.A. Teale. 2001. Resource partitioning and overlap in three sympatric species of *Ips* bark beetles (Coleoptera: Scolytidae). Oecologia. 128:443-453.

Billings, R. F, S. R. Clarke, M. V. Espino, C. P. Cordón, F. B. Meléndez, R. J. Campos, y G. Baeza. 2004. Gorgojo descortezador e incendios: una combinación devastadora para los pinares de América Central. Unasylva 217. 55: 15-21.

Birch, M.C., and P. Svhira. 1979. Novel approaches to forest insect control: exploiting olfactory interactions between species of Scolytidae, pp. 135-138. In W. E.Waters (ed.) Current Topics in Forest Entomology. John Wiley & Sons, New York.

Birch M.C., P. Svihra, T .D. Paine, J. C. Miller. 1980. Influence of chemically mediated behavior on host tree colonization by four cohabiting species of bark beetles. J. Chem. Ecol. 6:395-413.

Borden, J. H. 1974. Aggregation pheromones in the Scolytidae, pp. 135-160. In A. Neuberger, and E. L. Tatum (eds.), Frontiers of Biology. North Holland Publishing Company, Amsterdam.

Byers J. A. 1982. Male-specific conversion of the host plant compound, myrcene, to the pheromone, (+)-ipsdienol, in the bark beetle, *Dendroctonus brevicomins*. J. Chem. Ecol. 8: 363-371.

Byers, J.A., D. L. Wood,J .C. Craig, L.B. Hendry. 1984. Attractive and inhibitory pheromones produced in the bark beetle, *Dendroctonus brevicomis*, during host colonization: Regulation of inter- and intraspecific competition. J. Chem. Ecol. 10: 861–877.

Byers, J. A. 1989. Behavioral mechanisms involved in reducing competition in bark beetles. Holarct. Ecol. 12: 466-476.

Byers, J. A., and Q. Zhang. 2011. Chemical Ecology of Bark Beetles in Regard to Search and Selection of Host Trees, pp. 132-150. In T. Liu and L. Kang (eds). Recent advances in entomological research. Higher Education Press, Beijing.

Conn, J. E., Borden, J. H., Scott, B. E., Friskie, L. M., Pierce, H. D., and Oehlschkager, A. C. 1983. Semiochemicals for the mountain pinebeetle, Dendroctonus ponderosae, in British Columbia: field trapping studies. Can. J. For. 13:320-324.

Hofstetter, R. W., M. L. Gaylord, M. Martinson, and M. R. Wagner. 2012. Attraction to monoterpenes and beetle-produced compounds by syntopic *Ips* and *Dendroctonus* bark beetles and their predators. Agricultural and Forest Entomology. 14: 207–215.

Davis, T. S., and R. W. Hofstetter. 2009. Effects of gallery density and species ratio on the fitness and fecundity of two sympatric bark beetles (Coleoptera: Curculionidae). Environ. Entomol. 38: 639-650.

Hunt, D. W. A., J. H. Borden, H. D. Pierce, Jr. K. N. Slessor, G. G. S. King, E. K. Czyzewska. 1986. Sex-specific production of ipsdienol and myrcenol by *Dendroctonus ponderosae* exposed to myrcene vapors. J. Chem. Ecol. 12-1579-1586.

Hunt, D.W. A. and J. H. Borden. 1988. Response of mountain pine beetle, *Dendroctonus ponderosae* Hopkins, and pine engraver, *Ips pini* (Say), to ipsdienol in southwestern British Columbia. J. Chem. Ecol. 14: 277-293.

Lindgren, B. S. 1992. Attraction of Douglas-fir beetle, spruce beetle and a bark beetle predator (Coleoptera: Scolytidae and Cleridae) to enantiomers of frontalin. J. Entomol. Soc. B. C. 89: 13-17.

Midtgaard, F. and K. H. Thunes. 2002. Pine bark beetles in the Mountain Pine Ridge Forest Reserve, Belize: description of the species and advice on monitoring and combating the beetle infestations. Norwegian Forestry Group and the Inter-American Development Bank, Isadaltø, Norway.

Moreno, C. B. 2008. Diferenciación morfológica y comportamental de dos morfos de *Dendroctonus frontalis* Zimmermann (Coleoptera: Scolytinae) en Montebello Chiapas. M. S. Thesis. El Colegio Frontera Sur ECOSUR, Chiapas, México.

Moreno, B., J. Macías, B. T. Sullivan, and S. R. Clarke. 2008. Field response of *Dendroctonus frontalis* (Coleoptera: Scolytinae) to synthetic semiochemicals in Chiapas, Mexico. J. Econ. Entomol. 101:1821-1825.

Moser, J. C., B. A. Fitzgibbon, and K. D. 2005. The mexican pine beetle, *Dendroctonus mexicanus*: first record in the united states and co-occurrence with the Southern Pine Beetle, *Dendroctonus frontalis* (Coleoptera: Scolytidae or Curculionidae: Scolytinae). Entomological News. 116-4: 235-343.

Niño-Dominguez, A., B. T. Sullivan, J. H. López-Urbina, and J. E. Macías-Sámano. 2015. Pheromones-mediated mate location and discrimination by two syntopic sibling species of *Dendroctonus* barlk beetles in Chiapas, Mexico. J. Chem. Ecol. DOI 10.1007/s10886-015-0608-4.

Okland, B., O. Skarpaas, K. Kausrud. 2009. Threshold facilitations of interacting species. Popul. Ecol. 51:513-523.

Payne, T. L., J. V. Richerson, J. C. Dickens, J. R. West, K. Mori, C. W. Berisford, R. L. Hedden, J. P. Vité, and M. S. Blum. 1982. Southern pine beetle: Olfactory receptor and behavior discrimination of enantiomers of the attractant pheromone frontalin. J. Chem. Ecol. 8: 873-881.

Pureswaran, D. S., R. Gries, J.H. Borden, Jr. H. D. Pierce. 2000. Dynamics of pheromone production and communication in the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, and the pine engraver, *Ips pini* (Say) (Coleoptera: Scolytidae). Chemoecology. 10: 153–168.

Raffa, K. F. 2001. Mixed messages across multiple trophic levels: The ecology of bark beetle chemical communication systems. Chemoecology. 11: 49-65.

Rudinsky, J.A., M. E. Morgan, L. M. Libbey, and T. B. Putnam. 1974. Antiaggregative-rivalry pheromone of the Mountain Pine Beetle and ne arrestant of Southern Pine Beetle. Environ. Entomol. 3:90-98.

Seybold, S. J. 1993. Role of chirality in olfactory-directed behavior - Aggregation of pine engraver beetles in the genus *Ips* (Coleoptera, Scolytidae). J. Chem. Ecol. 19: 1809-1831.

Schlyter, F., and O. Anderbrant. 1993. Competition and niche separation between two bark beetles: existence and mechanisms. Oikos. 68:437-447.

Six, D. L., and R. Bracewell. 2015. *Dendroctonus*. Pp. 305-350. In F. E. Vega and R. Hopsthetter R (eds). Bark Beetles Biology and Ecology of Native and Invasive Species. Elservier press, Oxford UK.

Sullivan, B. T., M. J. Dalusky, D. Wakarchuk, and C. W. Berisford. 2007. Field Evaluations of Potential Aggregation Inhibitors for the Southern Pine Beetle, *Dendroctonus frontalis* (Coleoptera: Curculionidae). J. Entomol. Sci. 42: 139-149.

Sullivan, B.T., A. Niño, B. Moreno, C. Brownie, J. Macías-Sámano, S.R. Clarke, L. K. Kirkendall, G. Zúñiga. 2012. Biochemical evidence that *Dendroctonus frontalis* consists of two sibling species in Belize and Chiapas, Mexico. Ann Entomol. Soc. Am. 105:817-831.

Sullivan, B. T. and K. Mori. 2009. Spatial displacement of release point con enhance activity of an attractant pheromone synergist of a bark beetle. J. Chem. Ecol. 35-10: 1222-1233.

Sullivan, B. T., M. Dulusky, K. Mori, and C. Brownie. 2011. Variable Responses by Southern Pine Beetle, *Dendroctonus Frontalis* Zimmermann, to the Pheromone Component Endo-Brevicomin: Influence of Enantiomeric Composition, Release Rate, and Proximity to Infestations. J. Chem. Ecol. 37(4):403-11.

Svihra, P., T. D. Paine, and M. C. Birch. 1980. Interspecific olfactory communications in southern pine beetles. Naturwissenschaften. 67:518-520.

Svihra, P. 1982. Influence of opposite Sex on attraction produced by pioneer sex of four bark beetle species cohabiting pine in the Southern United States. J. Chem. Ecol. 8: 373-378.

Vité, J. P., R. F. Billings, C. W. Ware, and K. Mori. 1985. Southern pine beetle: Enhancement or inhibition of aggregation response mediated by enantiomers of *endo*-brevicomin. Naturwissenschaften. 72: 99-100.

Wagner, T. L., R. O. Flamm, and R. N. Coulson. 1985. Strategies for cohabitation among the southern pine bark beetle species: comparisons of life-process biologies. USDA For. Ser. GTR SO. 56: 87-101.

Wood, D. L., L. E. Browne, B. Ewing, K. Lindahl, W. D. Bedard, P. E. Tilden, K. Mori, G. B. Pitman, and P. R. Hughes. 1976. Western pine beetle - Specificity among enantiomers of male and female components of an attractant pheromone. Science 192: 896-898.

Wood, S. L. 1982. The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae), a taxonomic monograph. Great. Basin. Nat. Mem. 6.

Zúñiga, G., R. Cisneros, Y. Salinas-Moreno. 1995. Coexistencia de *Dendroctonus frontalis* ZIMMERMAN y *D. mexicanus* HOPKINS (Coleoptera: Scolytidae) sobre un mismo hospedero. Acta Zool. Mex. 64:59-62.

Table 1. Specifications of lures for field trapping experiments

Semiochemical	Abreviation	Experiment	Source[a]	Purity[b]	Chirality	Release device	Release rate (mg/d)[c]	Temp (°C)[d]
Pheromone component								
Frontalin	F	1, 2	Synergy	>95%	racemic	microcentri-fuge tube	2.5	23
		3A, 3B	Synergy	>98%	racemic	microcentri-fuge tube	6	22-24
endo-Brevicomin	E	1, 2	Synergy	>95%	racemic	bubble-cap	0.4-0.8	22-24
		3A, 3B	Synergy	93%	racemic	flexlure	0.5	25
Ipsdienol	I	1, 2	Synergy	93%	racemic	bubble-cap	0.7	25
		3A, 3B	Contech	97%	racemic	bubble-cap	1.6	25
		3A, 3B	Contech	>96%	(+3/-97)	bubble-cap	1.6	25
		3A, 3B	Contech	>96%	(+97/-3)	bubble-cap	1.6	25
Host volatiles								
Turpentine[e]	T	1, 2, 3A, 3B	Pinosa	-	-	Wick- bottle[f]	4 g/d	25

[a] Synergy Semiochemicals Corp., Burnaby, BC; Contech Enterprises (now Scotts), Victoria, BC; Pinosa Inc., Morelia, México.
[b] As provided by manufacturer.
[c] As provided by manufacturer or measured gravimetrically by the authors.
[d] Temperature at which release rate measurements were made.
[e] Turpentine was steam-distilled commercially from *Pinus oocarpa* Schiede ex Schltdl.
[f] Wick bait consisted of a filled 150 ml amber glass bottle with a cotton lamp wick extending 2 cm above the cap.

Table 2. Mean[a] (±SE) catches of *D. frontalis* and *D. mesoamericanus* (per trap/day) in funnel traps baited with combinations of synthetic semiochemicals during November 2013 at Parque Nacional Lagunas de Montebello, Chiapas, Mexico (experiment 2).

	D. frontalis		*D. mesoamericanus*	
Lure[b]	Females	Males	Females	Males
T	0.00 ± 0.00 (0)c	0.00 ± 0.00 (0)c	0.00 ± 0.00 (0)	0.00 ± 0.00 (0)
T+F	0.04 ± 0.04 (1)c*	0.52 ± 0.19 (14)bc	0.04 ± 0.04 (1)	0.00 ± 0.00 (0)
T+I	0.00 ± 0.00 (0)c	0.00 ± 0.00 (0)c	0.04 ± 0.04 (1)	0.04 ± 0.04 (1)
T+E	0.04 ± 0.04 (1)c	0.07 ± 0.05 (2)c	0.00 ± 0.00 (0)	0.04 ± 0.04 (1)
T+F+I	0.00 ± 0.00 (0)c	0.07 ± 0.05 (2)c	0.04 ± 0.04 (1)	0.04 ± 0.04 (1)
T+F+E	1.70 ± 0.50 (46)a*	5.00 ± 0.90 (136)a	0.04 ± 0.04 (1)	0.07 ± 0.05 (2)
T+E+I	0.04 ± 0.04 (1)c	0.04 ± 0.04 (1)c	0.00 ± 0.00 (0)	0.00 ± 0.00 (0)
T+F+E+I	0.30 ± 0.10 (8)b	0.89 ± 0.38 (24)b	0.00 ± 0.00 (0)	0.04 ± 0.04 (1)
F+E+I	0.00 ± 0.00 (0)c	0.22 ± 0.10 (6)bc	0.00 ± 0.00 (0)	0.00 ± 0.00 (0)

[a] Numbers in parentheses indicate raw insect catches. Within sex and species, treatments associated with the same lower-case letter did not differ significantly (GLM with a Poisson distribution and logarithmic function followed by a Wald test for pairwise comparisons between treatments within sex; α=0.05), and an asterisk indicates significant differences in catches between the two sexes (Mann-Whitney test, α=0.05). No significant treatment effects were detected for *D. mesoamericanus.*

[b] T=turpentine, F=frontalin, I= ipsdienol, E= *endo*-brevicomin.

Table 3 Mean[a] (±SE) catches of *D. frontalis* and *D. mesoamericanus* (per trap/day) in funnel traps baited with combinations of synthetic semiochemicals during November 2013 in Parque Nacional Lagunas de Montebello, Chiapas, Mexico (experiment 3).

	D. frontalis		*D.mesoamericanus*	
	female	male	female	male
Experiment 3A				
T+F+E[b]	1.5 ± 0.6 (18)a	3.5 ± 1.0 (42)a	0.2 ± 0.1 (2)	0.2 ± 0.1 (2)ab
T+F+E+I (±)	0.0 ± 0.0 (0)b	0.7 ± 0.3 (8)b	0.2 ± 0.2 (2)	0.0 ± 0.0 (0)b
T+F+E+I (+)	0.2 ± 0.1 (2)bc	0.4 ± 1.5 (5)b	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)b
T+F+E+I(-)	0.8 ± 0.4 (9)c	3.3 ± 1.5 (40)a	0.0 ± 0.0 (0)	0.4 ± 0.2 (5)a
Experiment 3B				
T+F+E (±)+I (±)	0.1 ± 0.1 (1)	0.2 ± 0.2 (2)	0.1 ± 0.1 (1)	0.2 ± 0.2 (2)
T+F+E (+)+I (±)	0.2 ± 0.2 (2)	0.4 ± 0.4 (4)	0.0 ± 0.0 (0)	0.2 ± 0.2 (2)
T+F+E (±)+I (+)	0.1 ± 0.1 (1)	0.8 ± 0.8 (7)	0.2 ± 0.2 (2)	0.0 ± 0.0 (0)

[a] Numbers in parentheses indicate raw insect catches. Within sexes and species, treatments associated with the same letter did not differ significantly [GLM with a negative binomial or Poisson distribution (in experiments 3A and 3B, respectively) with a logarithmic function followed by a Wald test for comparisons between treatments within sex; α=0.05]. No significant treatment effects were detected for either species in experiment 3B.

[b]T=turpentine, F=frontalin, I=ipsdienol, E=*endo*-brevicomin.

Figure Captions

Fig. 1. Numbers of *D. frontalis* caught in funnel traps during April-May 2013 in Parque Nacional Lagunas de Montebello, Chiapas, Mexico (experiment 1). Lures were combinations of frontalin (F), ipsdienol (I), *endo*-brevicomin (E), and turpentine (T). Within sex, treatments associated with the same lower-case letter did not differ significantly. Treatment effects were analyzed with GLM using a negative binomial distribution and a logarithmic function; a Wald test was used for pairwise comparisons between treatment means within each sex (α=0.05). An asterisk associated with a particular lure combination indicates a significant difference in response between the sexes (Mann-Whitney test, α=0.050).

Fig. 2. Numbers of *D. mesoamericanus* caught in funnel traps during April-May 2013 in Parque Nacional Lagunas de Montebello, Chiapas, Mexico (experiment 1). Lures were combinations of frontalin (F), ipsdienol (I), *endo*-brevicomin (E), and turpentine (T). Statistical analysis is same as in figure 1. Since there was not a sex by treatment interaction, sexes were combined. Treatments associated with the same lower-case letter did not differ significantly.

Fig. 3. Numbers of *D. frontalis* caught in traps baited with either female-infested or uninfested logs during December 2013 in Parque Nacional Lagunas de Montebello, Chiapas, Mexico (experiment 4). Bait logs were each infested with 30 females of either species; uninfested control logs had 30 mechanical drill holes but no insects. Numbers in parentheses indicate raw catches. There was not a significant treatment by sex interaction therefore sexes were combined for analyses (GLM with a Poisson distribution and logarithmic function followed by a Wald test for pairwise comparisons between treatments

within sex; α=0.05). Treatments labelled with the same lower-case letter did not differ significantly.

Fig. 4. Numbers of *D. mesoamericanus* caught in traps baited with either female-infested or uninfested logs during December 2013 in Parque Nacional Lagunas de Montebello, Chiapas, Mexico (experiment 4). Details as in figure 3. An asterisk indicates a significant difference between sexes in their response to the indicated treatment (Mann-Whitney test, α= 0.050).

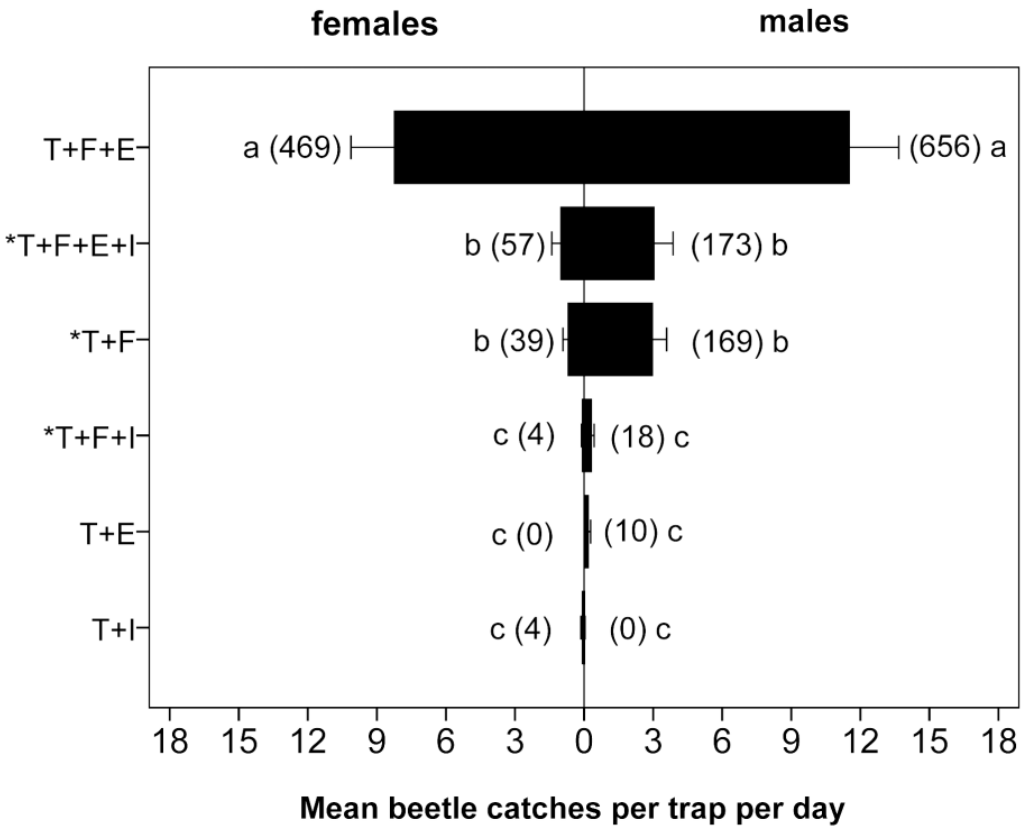

Figure 1.

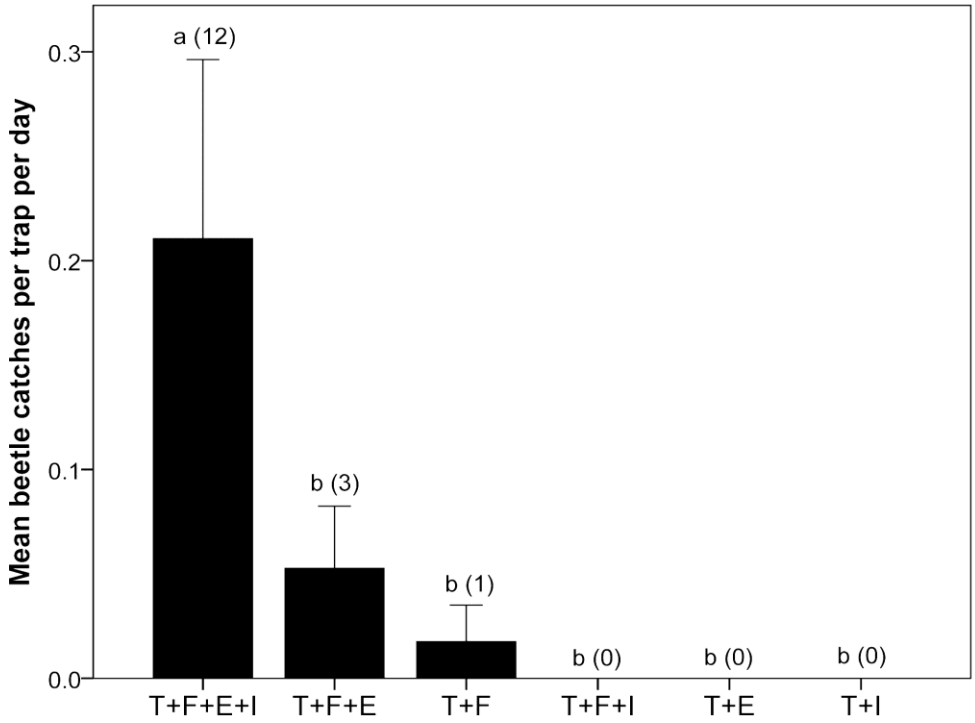

Figure 2.

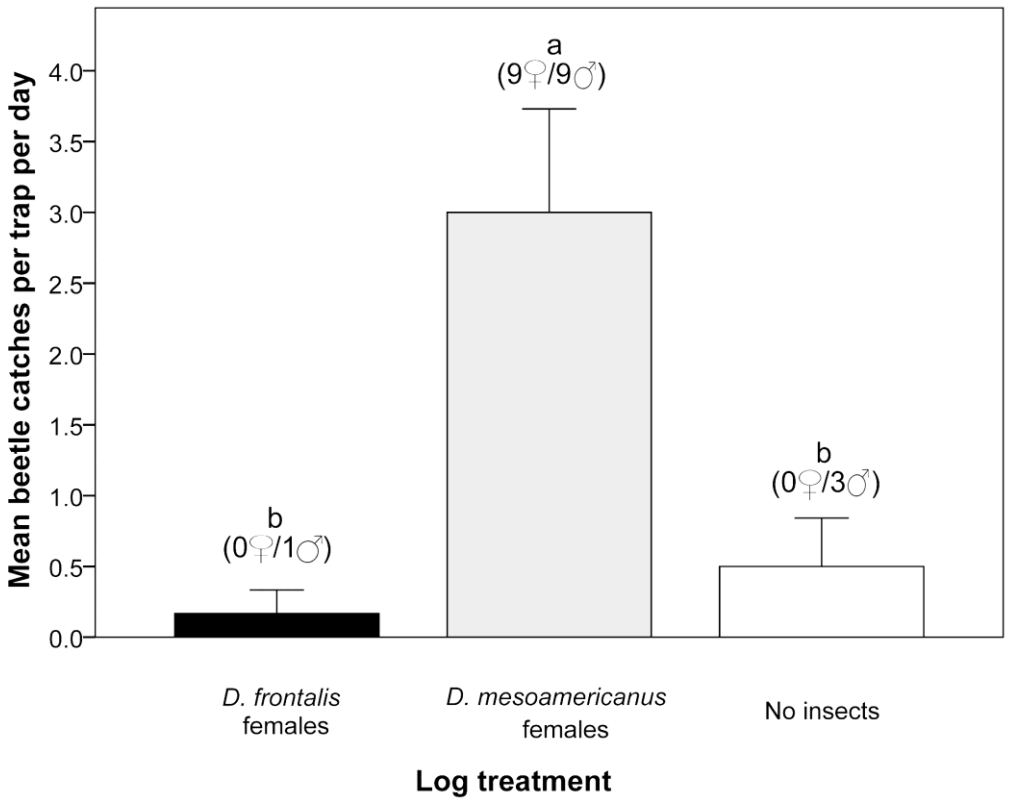

Figure 3.

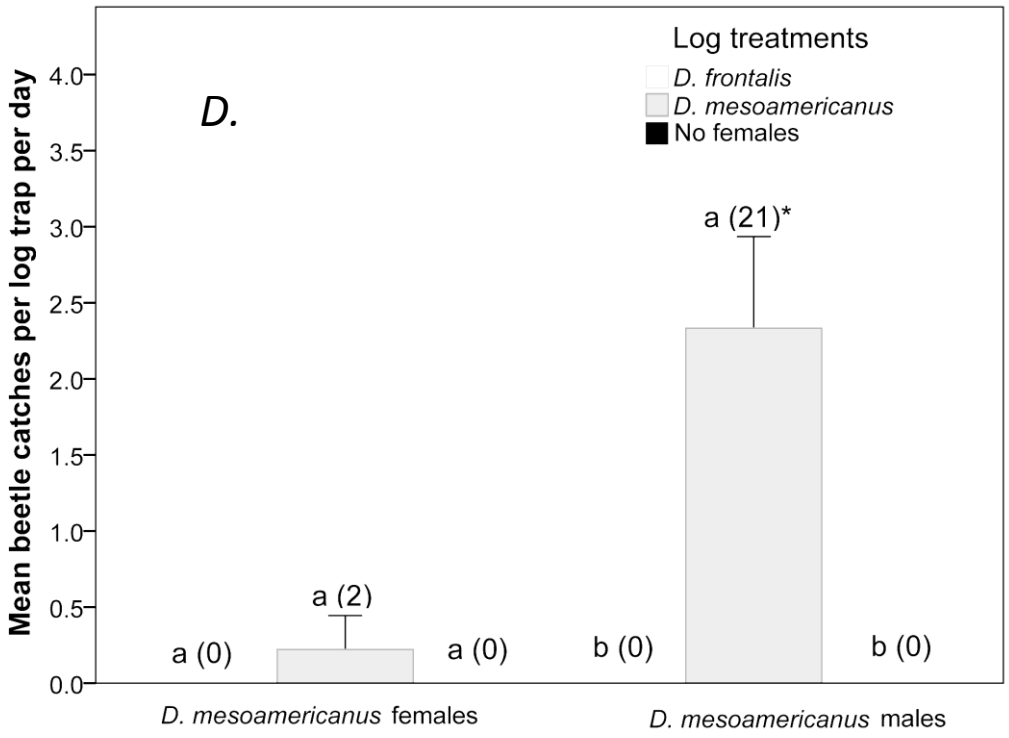

Figure 4.

CAPÍTULO V

Discusión general

Desde la emergencia de adultos hasta el establecimiento de la progenie en un nuevo hospedero los descortezadores llevan a cabo diversas actividades como el reconocimiento del sitio de agregación y la búsqueda de la pareja (Wood, 1982, Coulson and Klepzig, 2011), en las cuales está involucrada la comunicación sensorial olfativa. Ambos sexos de *D. frontalis* y *D. mesoamericanus* contribuyen con la liberación de feromonas, cuya nube de olor resulta ser muy similar entre las especies, un hecho interesante tratándose de especies hermanas que comparten el mismo hospedero. No obstante, los resultados obtenidos sugieren que *D. frontalis* y *D. mesoamericanus* difieren en el uso de los semioquímicos que cada especie produce, lo que parece depender de la actividad que se encuentren realizando. *D. frontalis* por ejemplo, en las pruebas que representaron el sitio de agregación (p.ej. uso de trampas cebadas con feromonas sintéticas o trampas-troza) la respuesta fue guiada por las feromonas relacionadas al sitio de agregación donde ambos sexos arriban y producen feromonas (p.ej. las hembras producen frontalina y los machos *endo*-brevicomina), lo cual concuerda con previos reportes (Sullivan y Mori, 2009, Sullivan, 2010). Mientras que la búsqueda de la pareja al parecer se lleva a cabo después del arribo al hospedero, dado que machos ambulantes de *D. frontalis* presentaron una fuerte atracción a los semioquímicos (frontalina) relacionados únicamente a la hembra conespecífica, mientras que la *endo*-brevicomina redujo la atracción de la frontalina.

En *D. mesoamericanus* el uso de los semioquímicos parece no funcionar en el mismo contexto en el que se ha sugerido para *D. frontalis*. Al parecer para *D. mesoamericanus* el reconocimiento de semioquímicos es más específica o restringida, al

menos en la orientación al sitio de agregación y en la búsqueda de la pareja, ya que *D. mesoamericanus* mostró, en la mayoría de las pruebas (p. ej. pruebas de trampeo en campo y de machos ambulantes en laboratorio), atracción significativa a los volátiles relacionados a las hembras conespecíficas. Aunado a lo anterior, *D. mesoamericanus* coloniza la parte inferior del fuste del hospedero, por lo que es probable que los adultos en vuelo al reconocer los semioquímicos relacionados a la hembra conespecífica definan con ello el sitio de colonización y la sección del fuste previo a su arribo en el hospedero. Posterior al arribo, al parecer la especificidad a los semioquímicos de la hembra permanece con este cambio de actividad (caminar), esto es cierto en machos ambulantes ensayados en laboratorio donde se obtuvo una respuesta significativa a volátiles relacionados a la hembra conespecífica.

La respuesta diferencial de una especie o entre especies a uno o más semioquímicos está condicionada al contexto en el que se encuentren (Wyatt, 2003). En *Pityogenes bidentatus* (Herbst) se ha reportado un comportamiento dicotómico a los monoterpenos del hospedero, evitando estos durante su orientación al sitio de agregación e ignorando su concentración cuando ha arribado al hospedero (Byers, 2012). En la repartición del recurso, *Ips grandicollis* (Heichhopff) e *Ips pini* Say presentan respuesta diferencial cuando a la feromona de agregación se añade los monoterpenos del hospedero siendo sinergista para una especie e inhibitoria para la otra, respectivamente (Erbilgin y Raffa, 2000). El uso diferencial de los semiquímicos entre *D. frontalis* y *D. mesoamericanus* parece ser una respuesta adaptativa en el contexto de la repartición del recurso y el aislamiento reproductivo, expresado probablemente en la especialización del sistema olfativo de los machos.

Las antenas son los principales órganos olfativos en la mayoría de los invertebrados (Greenfield, 2002), en insectos descortezadores forman un mazo en el extremo distal, en algunas especies como *I. trypographus* (Linnaeus), *D. valens* LeConte o *D. frontalis* el mazo antenal presenta bandas horizontales formadas por numerosas estructuras olfativas llamadas sensilas a través de las cuales son captadas las moléculas de olor (Payne et al., 1973). Recientemente se ha revelado que las sensilas de numerosas especies de descortezadores funcionan como unidades con interacción entre neuronas receptoras de olor, debido a que presentan dos o más de ellas especializadas a un solo compuesto, lo que genera interacción entre las neuronas ya que pueden estar combinadas entre neuronas especificas a feromonas y volátiles del hospedero o no hospedero, además una neurona puede inhibir la respuesta de la neurona vecina al presentarse el estímulo olfativo (Anderson, 2012). En cada especie de insectos descortezadores existe diferente distribución y agrupamiento de las sensilas en las bandas sensoriales lo que parece estar relacionado con la especialización de los semioquímicos y a su función ecológica (Anderson et al., 2009). Así, el sistema olfativo entre las especies de descortezadores puede estar formado por sensilas con una combinación única de neuronas especializadas que en conjunto pueden transmitir diferentes mensajes al cerebro del insecto (Leal, 2012). Por lo que es probable que diferentes códigos de olor puedan ser creados entre las especies y de esta manera presentar respuestas comportamentales diferenciales inclusive a los mismos estímulos olfativos (Galizia y Rössler, 2010).

El mazo antenal de *D. frontalis* posee al menos 3 tipos de sensilas, basiconicas y tricoidea II y III, de las cuales las de tipo basiconicas y tricoidea II son reportadas con función quimiorreceptoras, estás pueden presentar una, dos o múltiples neuronas receptoras de olor y son estimuladas por las feromonas relacionadas a su especie, es decir,

a la frontalina, *exo* y *endo*-brevicomina, verbenona, *trans*-verbenol y a los monoterpenos α-pineno y δ-3-careno (Dickens y Payne, 1978). Sin embargo no existe aún mayor conocimiento sobre el funcionamiento de las neuronas y su interacción dentro de cada tipo de sensila para dilucidar su respuesta comportamental a los semioquímicos. *D. mesoamericanus* ha sido recientemente descrita y no existe aún estudios referentes a la estructura y función de sus sensilas. Sin embargo, como un trabajo pionero se obtuvo que entre estas dos especies existe diferencias en el número de sensilas y su distribución en el mazo antenal (Armendaríz-Toledano y colaboradores, datos sin publicar) sugiriendo que esta divergencia pudiera permitir a las dos especies el uso diferencial de los mismos semioquímicos emitidos durante el proceso de colonización en el cual ambas especies están presentes (Anderson, 2012).

Debido a que la combinación de los diferentes componentes feromonales así como al índice de liberación y a su composición enantiomérica forman parte del código de comunicación entre las especies, la producción diferencial de feromonas ha sido utilizada como un carácter para estudios filogenéticos de las especies de descortezadores (Symonds y Elgar, 2004a). Sin embargo, entre las especies del género *Dendroctonus* se ha establecido que la producción de feromonas no representa un atributo para la segregación de grupos filogenéticos (Symons y Elgar, 2004b). No obstante, la comunicación química entre las especies que utilizan la olfacción, está basada no sólo en la producción de feromonas, si no en la co-evolución entre el emisor para la producción y el receptor para su interpretación (Greenfield, 2002), donde éste culmina con la respuesta comportamental. Es probable que la inclusión de estudios sobre el mecanismo y función del sistema olfativo del receptor, incluyendo pruebas electrofisiológicas y de comportamiento, pudiera favorecer el estudio filogenético de las especies de este género, debido a que cada especie posee su

propio código de olor que les permite diferenciarse como especie, tal y como lo sugieren los resultados obtenidos en este trabajo, donde las hembras produjeron diferentes feromonas y los machos respondieron a esta diferencia, lo que implica que aún con similar producción de feromonas entre las especies de *Dendroctonus*, éstas logran distanciarse mediante el reconocimiento diferencial de las feromonas (Lindgren y Miller, 2002).

Lo anterior permite confirmar que entre estas dos especies operan mecanismos de aislamiento reproductivo de tipo precopulatorio mediado por semioquímicos. No obstante la respuesta de atracción a feromonas de hembras heteroespecíficas, aunque no significativa, sugiere que este mecanismo no funciona de manera absoluta para el aislamiento reproductivo entre las especies. Es probable que la combinación de otros sistemas de comunicación pudieran funcionar en conjunto para reforzar el aislamiento reproductivo, lo cual es común entre las especies simpátricas cercanamente relacionadas (Coyne y Orr, 2004). Tal y como lo sugieren las pruebas preliminares realizadas por Sullivan (datos sin publicar) quién encontró diferencias en los sonidos producidos de la estridulación de machos adultos cerca de hembras conespecíficas. Adicionalmente existen otros aspectos bio-ecológicos que pudieran contribuir en el aislamiento reproductivo tales como: 1) la distribución espacial que actualmente presentan las especies a lo largo del hospedero con una sección de sobrelapamiento (Moreno, 2008), 2) la emergencia diferencial de la progenie de cada especie (que sugiere arribos o ciclos de vida diferentes) (Niño, datos sin publicar), 3) la aparente inexistencia de intercruzas naturales, pero posibles en laboratorio (lo que sugiere aislamiento postzigotico incompleto) (Armendáriz- Toledano et al., 2014), y 5) diferente fórmula kariológica entre las especies (Armendáriz-Toledano et al., 2014).

Estos atributos representan un campo de estudio interesante para dilucidar el hecho de que estas dos especies coexisten y de cómo logran permanecer segregadas como identidades diferentes, donde probablemente la selección natural, selección disruptiva (Barton y Rodríguez de Cara, 2009) y el reforzamiento (Noor, 1999, Bracewell et al., 2010) en simpatría pudieron ser las fuerzas evolutivas que promovieron los atributos observados actualmente entre estas dos especies que les permite coexistir y mantener un aislamiento reproductivo (Coyne y Orr, 2004).

Conclusiones generales

Las pruebas realizadas permitieron establecer que los semioquímicos son actualmente mediadores del aislamiento reproductivo entre las especies de *D. frontalis* y *D. mesoamericanus,* donde la producción de *endo*-brevicomina e ipsdienol por la hembra de *D. mesoamericanus* conduce el reconocimiento de la pareja entre los machos de ambas especies, los cuales presentan diferentes estrategias para el reconocimiento de la pareja durante la colonización del hospedero.

D. frontalis reconoce a la pareja en primer lugar reconociendo a la distancia el sitio de agregación y posterior al arribo se efectúa el reconocimiento formal de la pareja. *D. mesoamericanus* es especifico en reconocer a la pareja a la distancia y posterior al arribo en el fuste del hospedero.

El mecanismo de aislamiento reproductivo mediado por semioquímicos entre estas dos especies no es absoluto. Por lo que pudiera estar efectuándose a través de la combinación de más de un mecanismo de aislamiento. La preferencia del hábitat, presuntamente la alocronía discreta de arribo de *D. mesoamericanus* durante el periodo de apareamiento y

de una probable producción diferencial de sonido mediante estridulaciones pudieran ser explorados como atributos de mecanismos de aislamiento reproductivo que confirmen el reforzamiento del aislamiento reproductivo precigótico mediado por semioquímicos.

Finalmente este trabajo ofrece importantes directrices sobre el uso de semioquímicos para el monitoreo de descortezadores, básicamente en aquellos sitios donde *D. frontalis* y *D. mesoamericanus* coexisten, para lo cual es factible añadir al atrayente de *D. frontalis* (α-pineno y frontalina) las feromonas (±) *endo*-brevicomina e (±) ipsdienol, cuya combinación permitiría la captura de ambas especies y su representación numérica en planes de monitoreo a largo plazo de estas especies.

LITERATURA CITADA

Amarasekare P. 2002. Interference Competition and Species Coexistence. Biol. Sciences. 269: 2541-2550.

Andersson MN. 2012. Mechanisms of Odor Coding in Coniferous Bark Beetles: From Neuron to Behavior and Application. Psyche. 1:14. Doi:10.1155/2012/149572.

Andersson MN, Larsson MC, y Schlyter F. 2009. Specificity and Redundancy in the Olfactory System of the Bark Beetle *Ips typographus*: Single-cell Responses to Ecologically Relevant Odors. J. Insect Physiology. 55:556–567.

Armendáriz-Toledano F, Niño A, Sullivan BT, Macías-Sámano J, Victor J, Clarke SR, and Zuñiga G. 2014. Two Species within *Dendroctonus frontalis* (Coleoptera: Curculionidae): Evidence from Morphological, Karyological, Molecular, and Crossing Studies. Ann. Entomol. Soc. Am. 107:11-27.

Armendáriz-Toledano F, Niño-Domínguez A, Sullivan BT, Kirkendall LR, and Zúñiga G. 2015. A new Species of Bark Beetle, *Dendroctonus mesoamericanus* sp. nov. (Curculionidae: Scolytinae) in Southern Mexico and Central America. Ann. Entomol. Soc. Am 1-12.

Barton NH y Rodríguez de Cara MA. 2009. The Evolution of Strong Reproductive Isolation. Evolution. 63: 1171–1190.

Beltman JB y Metz JA Jr. 2005. Speciation: More Likely Through a Genetic or Through a Learned Habitat Preference?. Proc. R. Soc. B. 272: 1455–1463.

Bentz JB, Régniére J, Fetting CJ, Hansen EM, Hayes JL, Hicke JA, Kelsey RG, Negrón JF, y Seybold SJ. 2010. Climate Change and Bark Beetles of the Western United States and Canada: Direct and Indirect Effects. BioScience. 60: 602-613.

Billings RF, Clarke SR, Espino-Mendoza V, Cordón-Cabrera P, Meléndez-Figueroa B, Ramón-Campos J, y Baeza G. 2004. Gorgojo Descortezador e Incendios: Una Combinación Devastadora para los Pinares de América Central. UNASYLVA, 55: 15-19.

Birch MC, Svihra P, Paine TD, y Miller JC. 1980. Influence of Chemically Mediated Behavior on Host Tree Colonization by Four Cohabiting Species of Bark Beetles. J. Chem. Ecol. 6:395-413.

Bolnick DI y Fitzpatrick BM. 2007. Sympatric Speciation: Models and Empirical Evidence. Annu. Rev. Ecol. Evol. Syst. 38:459–87.

Bolnick DI. 2004. Waiting for Sympatric Speciation. Evolution, Vol. 58 (4): 895-899.

Bracewell RR, Pfrender ME, Mock KE, y Bentz BJ. 2010. Cryptic Postzygotic Isolation in an Eruptive Species of Bark Beetles (*Dendroctonus ponderosa*). Evolution 65: 961-975.

Browne LE, Wood DL, Bedard WD, Silverstein RM y West JR. 1979. Quantitative Estimates of the Western Pine Beetle Attractive Pheromone Components, *Exo*-brevicomin, Frontalin, and Myrcene in nature. J. Chem. Ecol. 5: 397-414.

Byers JA.1989. Chemical Ecology of Bark Beetles. Experientia. 45: 271–283.

Byers JA. 1999. Effects of Attraction Radius and Flight Paths on Catch of Scolytid Beetles Dispersing Outward Through Rings of Pheromone Traps. Journal of Chemical Ecology. 25: 985–1005.

Byers JA. 2012. Bark Beetles, *Pityogenes bidentatus*, Orienting to Aggregation Pheromone Avoid Conifer Monoterpene Odors When Flying but NotWhenWalking. Psyche, ID 940962: 1-10.

Byers JA y Zhang Q. 2011. Chemical Ecology of Bark Beetles in Regard to Search and Selection of Host Trees. pp. 132-150. In T. Liu and L. Kang (eds). Recent Advances in Entomological Research. Higher Education Press, Beijing.

CONAFOR. 2007. Salud Forestal. Anuario. Pp. 190-191. Liga web: http://148.223.105.188:2222/gif/snif_portal/administrator/sistemas/archivoslasdemas/1295030069_ANUARIO_2007.pdf.

Coulson RN y Klepzig KD. 2011. Southern Pine Beetle II. USDA Forest Service Southern Research Station General Technical Report SRS-140, pp 25-50.

Coyne JA, Kim SY, Chang AS, Lachaise D, y Elwyn S. 2002. Sexual Isolation Between Two Sibling Species with Overlapping Ranges: *Drosophila santomea* and *Drosophila yakuba*. Evolution. 56 (12): 2424–2434.

Coyne JA y Orr HA. 2004. Speciation. Ed. Sinauer Associates. Sunderland, Massachusetts. Pp. 545.

Davis TS y Hofstetter RW. 2009. Effects of Gallery Density and Species Ratio on the Fitness and Fecundity of Two Sympatric Bark Beetles (Coleoptera: Curculionidae). Environmental Entomology, 38(3), 639-650.

Denno RF, McClure MS, y Ott JR. 1995. Interspecific Interactions in Phytophagus Insects: Competition Reexamined and Resurrected. Annual Review of Entomology. 40: 297-331.

Dickens JC y Payne TL. 1978. Structure and Function of the Sensilla on the Antennal Club of the Southern Pine Beetle, *Dendroctonus frontalis* (Zimmerman) (Coleoptera: Scolytidae). Int. J. Insect MorphoL & Embryol. 7: 251-265.

Drés M y Mallet J. 2002. Host Races in Plant-Feeding Insects and Their Importance in Sympatric Speciation. Biological Sciences. Vol. 357 (1420): 471-492.

Erblingin N y RAFFA K F. 2000. Opposing Effects of Host Monoterpenes on Responses by Two Sympatric Species of Bark Beetles to Their Aggregation Pheromones. J. Chem. Ecol. 26:2527-2548.

Fettig CJ. 2012. Forest Health and Bark Beetles. In: North M. (Ed.), Managing Sierra Nevada Forests. U.S. Department of Agriculture, Forest Service, Albany, CA. pp. 13–22. General Technical Report PSWGTR-237.

Fiedler CE y McKinney ST. 2004. Forest Structure, Health, and Mortality in Two Rocky Mountain Whitebark Pine. Natural Areas Journal. 34:290-299.

Friesen VL, Gómez-Díaz E, Bolton M, Furness RW, González-Solís, y Monteiro LR. 2005. Sympatric Speciation by Allochrony in a Seabird. PNAS. Vol. 104 (47): 18589–18594.

Futuyma DJ y Peterson SC. 1985. Genetic Variation in the Use of Resources by Insects. Ann. Rev. Entomol. Vol. 30: 217-38.

Gara RI. 1967. A Field Olfactometer for Studying the Response of the Southern Pine Beetle to Attractants. J. Econ. Entomol. 60: 1180-1181.

Gara RI y Coster JE. 1968. Studies on the Attack of the Southern Pine Beetle. III. Sequence of Tree Infestation within Stands. Contrib. Boyce-Thomson Inst. 24:77-85.

Galizia GC and Rössler W. 2010. Parallel Olfactory Systems in Insects: Anatomy and Function. Ann. Rev. Entomol. 55:399-420.

Greenfield MD. 2002. Signalers and Receivers: Mechanisms and Evolution of Arthropod Communication. Oxford University Press. Pp. 282.

Haack RA, Eckelmann CM, y Green E. 2000. Southern Pine Beetle Outbreak in Belize. Michigan Entomol. Soc. Vol. 45: 13-15.

Hughes PR, Renwick JAA, y Vité JP. 1976. The identification and field bioassays of chemical attractants in the round headed pine beetle. Environmental Entomology. 5: 1165-1168.

Kelley ST y Farrell BD. 1998. Is Specializationa Dead End? The Phylogeny of Host Use in *Dendroctonus* Bark Beetles (Scolytidae).Evolution, Vol. 52. No. 6: 1731-1743.

Kinzer GW, Fentiman Jr A F, Page Jr TF, Foltz RL, y Vité JP. 1969. Bark Beetle Attractants: Identification, Synthesis and Field Bioassay of a New Compound Isolated from *Dendroctonus*. Nature 221: 477-478.

Lanier GN y Burkholder WE. 1974. Pheromones in Speciation of Coleopteran*. In* A Neuberger y EL Tatum (eds.). Frontiers of Biology. Vol. 32. North Holland (ed.). Amsterdam, London. Pp. 161-189.

Lanier G, Hendrichs JP, y Flores JE. 1988. Biosystematics of the *Dendroctonus frontalis* (Coleoptera: Scolytidae) Complex. Ann. Entomol. Soc. Am. 81: 403-418.

Leal WS. 2012. Odorant reception in insects: Roles of receptors, binding proteins, and degrading enzymes. Ann. Rev. Entomol. Vol. 58: 373-391.

Lindgren BS y Miller DR. 2002. Effect of Verbenone on Five Species of Bark Beetles (Coleoptera: Scolytidae) in Lodgepole Pine Forests. Environ. Entomol. 31: 759-765 (200).

Mayr E. 1963. Animal Species and Evolution. Belknap, Cambridge, MA.

McNulty SG y Aber JD. 2001. US National Climate Change assessment on Forest Ecosystems: An introduction. BioScience 51: 720–722.

Midtgaard F y Thunes KH. 2002. Pine Bark Beetles in The Mountain Pine Ridge Forest Reserve, Belize: Description of the Species and How to Monitor and Combat the Beetle Infestations. Isadaltø, Noruega, Grupo Forestal Noruego, Banco Interamericano de Desarrollo.

Moreno CB. 2008. Caracterización Morfológica y Comportamiento de Atracción a Feromonas Producidas por *Dendroctonus frontalis*. Tesis de Maestría de ECOSUR. 55 pp.

Morris DW. 2011. Adaptation and Habitat Selection in the Eco-evolutionary Process. Proc. R. Soc. B. Vol. 278: 2401–2411.

Moser JC, Fitzgibbon B, y Klepzig KD. 2005. The Mexican Pine Beetle, *Dendroctonus*: First Record in the United States and Co-occurrence with the Southern Pine Beetle-*Dendroctonus frontalis* (Coleoptera: Scolytidae or Curculionidae: Scolytinae). Entomol. News. 116 (4): 235-243.

Niño-DomínguezA. 20007. Índice Enantiomérico y Cuantificación de las Feromonas Frontalina y *endo*- brevicomina de *D. frontalis* (Coleoptera: Curculionidae), en Montebello, Chiapas. Tesis de Maestría de ECOSUR. Pp. 300.

Noor MA F. 1999. Reinforcement and other Consequences of Sympatry. Heredity. 83:503-508.

Okland B, Skapaas O, y Kausrud K. 2009. Threshold Facilitations of Interacting Species. Popul. Ecol. 51:513-523.

Orr HA y Turelli M. 2001. The Evolution of Postzygotic Isolation: Accumulating Dobzhansky-Muller Incompatibilities. Evolution. Vol. 55 (6): 1085-1094.

Ortiz-Barrientos D, Counterman BA, y Noor MAF. 2004. The Genetics of Speciation by Reinforcement. PlosBiol. Vol. 2 (12): 2256-2263.

Payne TL, Moeck HA, Willson CD, Coulson RN y Humphreys WJ. 1973. Bark Beetle Olfaction- II. Antennal Morphology of Sixteen Species of Scolytidae (Coleoptera). Int. J. Insect. Morphol. & Embryol. 2:177-192.

Peterson MA, Honchak BM, Locke SE, Beeman TE, Mendoza J, Green J, Buckingham KJ, White MA, y Monsen KJ. 2005. Relative Abundance and the Species-Specific Reinforcement of Male Mating Preference in the Chrysochus (Coleoptera: Chrysomelidae) Hybrid Zone. Evolution, Vol. 59(12): 2639-2655.

Pitman GB y Vité JP. 1969. Aggregation Behavior of *Dendroctonus ponderosae* (Coleoptera: Scolytidae) in Response to Chemical Messengers. The Canadian Entomologist. 101:143-149.

Pitman GB, Vité JP, Kinzer GW, y Fentiman AF. 1969. Specificity of Population-Aggregating Pheromones in *Dendroctonu*s. J. Insect Physiol. Vol. 15: 363-366.

Price, CSC, Kim CH, Gronlund CJ, y Coune JA. 2001. Cryptic Reproductive Isolation in the *Drosophila simulans* Species Complex. Evolution. Vol. 55 (1): 81-92.

Pureswaran DS y Sullivan, TB. 2012. Semiochemical Emission from Individual Galleries of the Southern Pine Beetle, (Coleoptera: Curculionidae: Scolytinae), Attacking Standing Trees. J. Econ. Entomol. Vol. 105:140-148.

Renwick JAA.1967. Identification of two Oxygenated Terpenes from the Bark Beetles *Dendroctonus frontalis* and *Dendroctonus brevicomis*. Contributions from the Boyce Thompson Institute of Plant Research. 23:355.

Renwick JAA. y Vité JP. 1969. Bark Beetle Attractant: Mechanism of Colonization by *Dendroctonus frontalis*. Nature. 224:1222-1223.

Rivas LR. 1964. A Reinterpretation of the Concepts "Sympatric" and "Allopatric" with Proposal of the Additional Terms "Syntopic" and "Allotopic". Systematic Zoology, Vol. 13: 42-43.

Rudinsky JA, Morgan ME, Libbey LM, y Putnam TB. 1974. Additional Components of the Douglas-fir Beetle (Coleoptera: Scolytidae) Aggregative Pheromone and their Possible Utility in Pest Control. Zeitschrift für Angewandte Entomologie. 76:65-77.

Ryker LC y Libbey LM. 1982. Frontalin in the Male Mountain Pine Beetle J. Chem. Ecol. 8: 1399-1409.

Salinas-Moreno YG. Mendoza Correa, Barrios MA, Cisneros R, Macías-Sámano J, y Zúñiga G. 2004. Areography of the Genus *Dendroctonus* (Coleoptera: Curculionidae) in Mexico. J. Biogeography. 31:1163-1177.

Savage M. 1994. Anthropogenic and Natural Disturbance and Patterns of Mortality in a Mixed-Conifer Forest in California. Can JFor.Res. 24: 1149–1159.

Schoener TW. 1982. The Controversy Interspecific Competition. American Scientist. Vol. 70: 586-594.

Servedio MR. 2001. Beyond Reinforcement: the Evolution of Premating Isolation by Direct Selection on Preferences and Postmating, Prezygotic Incompatibilities. Evolution. Vol. 55 (10): 1909-1920.

Six DL, y Bracewell R. 2015. *Dendroctonus*. Pp. 305-350. In FE Vega and R Hofstetter (eds). Bark Beetles Biology and Ecology of Native and Invasive Species. Elservier press, Oxford UK.

Smadja C y Butlin RK. 2009. On the Scent of Speciation: The Chemosensory System and Its Role in Premating Isolation. Heredity. Vol. 102: 77–97.

Sturgeon KB y Mitton JB. 1982. Evolution of Bark Beetle Communities. *In* J B Mitton and K B Sturgeon (eds.). Bark Beetles in North American Conifers. University of Texas (ed.) Austin, Texas. Pp. 527.

Sullivan B T, Shepherd W P, Pureswaran D S, Táchira T, y Mori K. 2007. Evidence that (+)-*endo*-brevicomin is a Male-Produced Component of the Southern Pine Beetle Aggregation Pheromone. J. Chem. Ecol. 33: 1510-1527.

Sullivan BT y Mori K. 2009. Spatial Displacement of Release Point Can Enhance Activity of an Attractant Pheromone Synergist of a Bark Beetle. J Chem. Ecol., 35: 1222-1233.

Sullivan BT. 2010. Southern Pine Beetle Behavior and Semiochemistry. Pp. 25-50, in R N. Coulson and KD Klepzig. Southern Pine Beetle II. Gen. Tech Rep. SRS-140. Ashville, NC: U. S. Department of Agriculture Forest Service. Southern Research Station.

Sullivan BT, Niño A, Moreno B, Brownie C, Macias-Sámano J, Clarke SR., Kirkendall LK, y Zúñiga G. 2012. Biochemical Evidence that *Dendroctonus frontalis* Consists of Two Sibling Species in Belize and Chiapas, Mexico. Ann. Entomol. Soc. Am. 105:817-831.

Svihra P, Paine TD, y Birch MC. 1980. Interspecific Olfactory Communications in Southern Pine Beetles. Naturwissenschaften 67:518-520.

Symonds RE y Elgar MA. 2004a. Species Overlap, Speciation and the Evolution of Aggregation Pheromones in Bark Beetles. Ecology Letters. 7: 202–212.

Symonds RE y Elgar MA. 2004b.The mode of Pheromone Evolution: Evidence from Bark Beetles. Proc. R. Soc. Lond. B. Vol. 271: 839-846.

Tao Y y Hartl DL. 2003. Genetic Dissection of Hybrid Incompatibilities between *Drosophila simulans* and *D. mauritlana* III. Heterogeneous Accumulation of Hybrid Incompatibilities, Degree of Dominance, and Implications for Haldane`s rule. Evolution. Vol. 57 (11): 2580-2598.

Templeton AR.1980. Modes of Speciation and Inferences Based on Genetic Distances. Evolution. Vol. 34 (4): 719-729.

Vité JP y Renwick JAA. 1971. Inhibition of *Dendroctonus frontalis* Response to Frontalin by Isomers of Brevicomin. Naturwissenschaften 8: 418-419.

Wood SL.1982. The Bark and Ambrosia Beetles of North and Central America (Coleoptera: Scolytidae), A Taxonomic Monograph. Great Basin Nat. Mem. Vol. (6): pp. 1357.

Wyatt TD. 2003. Pheromones and Animal Behaviour. Cambridge University Press. Pp. 188.

Zúñiga G, Cisneros R y Salinas-Moreno Y, 1995. Coexistencia de *Dendroctonus frontalis* ZIMMERMAN y *D. mexicanus* HOPKINS (Coleoptera: Scolytidae) Sobre un Mismo Hospedero. Acta Zool. Mex. No. 64; 59-62.

Zúñiga G, Mendoza CG, Cisneros R, y Salinas-Moreno Y.1999. Zonas de Sobre Posición en las Aéreas de Distribución Geográfica de las Especies Mexicanas de *Dendroctonus* ERICHSON (Coleoptera: Scolytidae) y Sus Implicaciones Ecológico-Evolutivo. Acta Zool. Mex. No. 77: 1-22.