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The role of pheromones and temporal mechanisms in the reproductive isolation of *Monochamus maculosus*, *Monochamus notatus*, and *Monochamus scutellatus* (Coleoptera: Cerambycidae)

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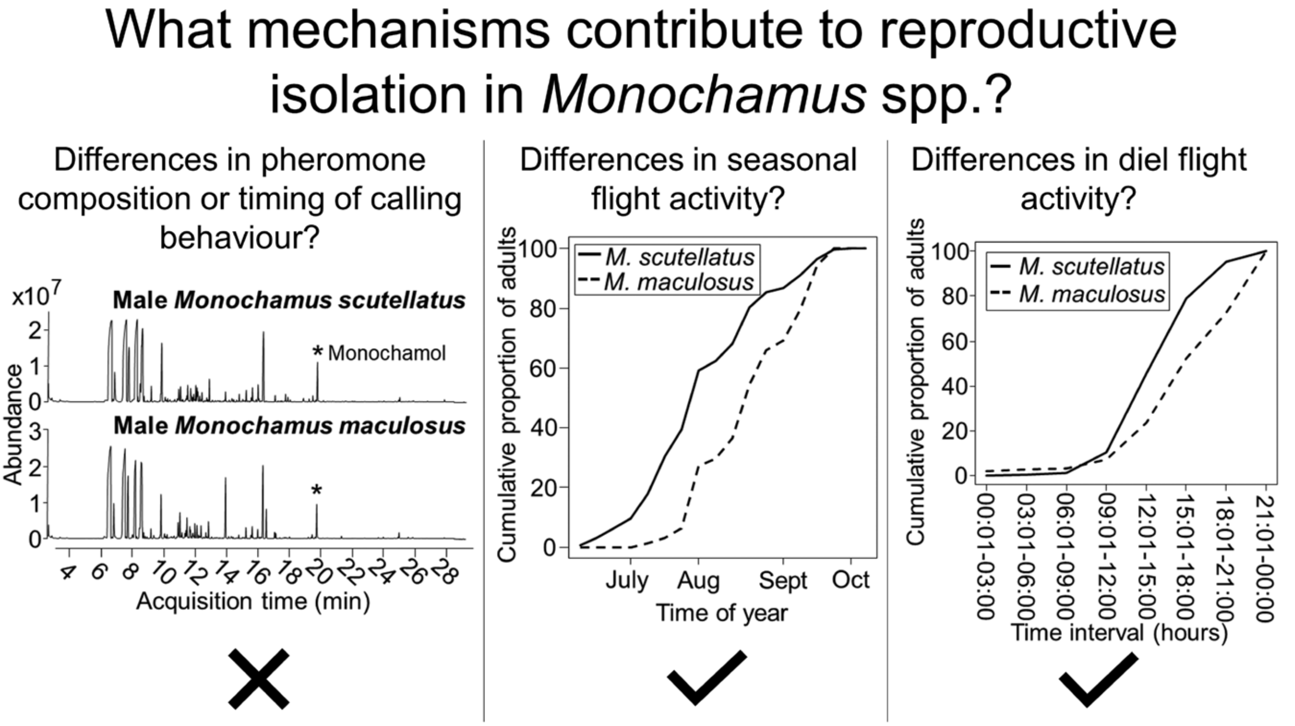
The mechanisms used to facilitate mate location among insects—such as pheromones—can inhibit interspecific attraction and confer reproductive isolation. However, pheromone components seem conserved within the genus *Monochamus* (Coleoptera: Cerambycidae) with the compound 2-(undecyloxy)ethanol (referred to as monochamol) being identified as the sex-aggregation pheromone or putative pheromone attractant for at least 15 species in this genus. This pheromone parsimony suggests the existence of additional isolating mechanisms. Here we describe the results of laboratory and field assays of additional mechanisms, including laboratory volatile collections and field trapping experiments that assessed the potential presence of additional pheromone components, diel rhythms of pheromone production, and phenological/diel flight activity in the reproductive isolation of sympatric populations of three species of *Monochamus* in the Great Lakes Forest Region of Ontario, Canada. Chemical analyses of volatile extractions indicate that monochamol is produced by male *Monochamus maculosus* and *Monochamus scutellatus*, but no qualitative differences were observed in male extracts of these two species suggesting that there are no additional pheromone components that confer specificity. No quantitative differences were found in the production of monochamol by male *M. scutellatus* during the photophase and scotophase suggesting there is no diel rhythm in pheromone production. Our results indicate that *M. scutellatus* flies earlier in the season and day than *M. maculosus* and *Monochamus notatus*, which could partially contribute to their reproductive isolation. Overall, no obvious differences in pheromone composition were observed but minor differences in flight times were observed, suggesting other isolating mechanisms may exist.

Keywords: mate attraction, flight phenology, prezygotic isolation, longhorned beetle.

Graphical Abstract



Introduction

One of the biggest challenges for species that reproduce sexually is finding a receptive member of the opposite sex. Effective mechanisms for locating and attracting mates should be favored since many insects that sexually reproduce have a short life-span and limited opportunities to reproduce (Foster 2005, Gullan and Cranston 2010). In some species adults do not feed and their sole function is reproduction [e.g., all Ephemeroptera and Ceratopogonidae (Diptera) (Gullan and Cranston 2010) and some species of Cerambycidae (Coleoptera) (Haack 2017)]. Therefore, traits that facilitate the rapid recognition, location, and attraction of receptive conspecifics are usually favored by natural selection (Bonduriansky 2011). In addition, heterospecific interactions during the process of mate acquisition are often associated with fitness reduction due to lost resources (e.g., energy, nutrients, gametes) associated with attempts to locate and mate with heterospecifics (Groot et al. 2006, Gröning and Hochkirch 2008). Heterospecific courtship can also involve substantial opportunity costs and predation/parasitism risks (Pervez and Omkar 2022). These costs should impose selection pressure favoring mechanisms that facilitate rapid and accurate mate location (Symonds and Elgar 2008, Smadja and Butlin 2009, Allison and Cardé 2016).

Species-specific signals are one of the mechanisms used by insects to communicate the location of receptive individuals to conspecifics. Among insects, olfactory signals such as pheromones are common (Greenfield 2002). Pheromones are chemicals produced and released by one individual that elicit a specific behavioral or physiological response in conspecifics (Karlson and Lüscher 1959) and are composed of one or more chemical compounds (Cardé and Baker 1984, Wicker-Thomas 2011). Qualitative and quantitative differences in a pheromone blend (i.e., differences in the absolute and relative amounts of pheromone components) contribute to species-specific mate recognition and confer reproductive isolation (Symonds and Elgar 2008, Yang et al. 2009, Allison and Cardé 2016).

Surprisingly, pheromone parsimony seems common in the Cerambycidae with identical or chemically similar pheromone components being shared within and across genera, tribes, and subfamilies (Hanks and Millar 2013, 2016, Millar and Hanks 2017). In the subfamily Lamiinae, an interesting case of pheromone parsimony includes species from the genus *Monochamus* Dejean. The compound 2-(undecyloxy)ethanol (monochamol) has been demonstrated to be a pheromone or putative pheromone attractant for multiple *Monochamus* species that are native to North America and Eurasia (Pajares et al. 2010, 2013, Teale et al. 2011, Allison et al. 2012, Fierke et al. 2012, Macias-Samano et al. 2012, Wickham et al. 2014, Ryall et al. 2015, Miller et al. 2016, Lee et al. 2017, 2018, Andrade et al. 2024). This compound serves as an aggregation -sex pheromone (sensu Cardé 2014) that is produced by males and attracts both sexes. Eight species of *Monochamus* are present in North America and some are sympatric and at least partially synchronic, including *Monochamus maculosus* Haldeman, *Monochamus notatus* Drury, and *Monochamus scutellatus* Say, in Ontario, Canada (Bousquet et al. 2017, EFSA PLH Panel 2018). These 3 *Monochamus* species attack severely stressed or recently dead trees in the genera *Pinus* L., *Picea* A. Dietr., and *Abies* Mill. (Pinales: Pinaceae) (Akbulut and Stamps 2011, Akbulut et al. 2017). Females lay eggs in the phloem of host trees through crevices or oviposition slits in the bark, where, after hatching, larvae feed before excavating deep U-shaped galleries into the sapwood for pupation. These species are considered secondary pests as larval tunneling downgrades wood value and results in significant losses (Safranyik & Raske 1970, Gardiner 1975, Allison et al. 2004). In addition, *Monochamus* species act as primary vectors of the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle (Rabditida: Aphelenchoididae), the causal agent of pine wilt disease (Wingfield 1987, Sousa et al. 2001, Naves et al. 2016).

Although the observed pheromone parsimony in *Monochamus* species makes them vulnerable to interspecific attraction, there is limited evidence that hybridization occurs in the group (pers. comm. in Hughes and Hughes 1987). Differences in seasonal and/or diel activity patterns in pheromone production may contribute to reproductive isolation in *Monochamus* by reducing the potential for cross-attraction to shared pheromone components. This mechanism has been shown to serve as a segregating mechanism in some sympatric species of Cerambycidae (Hanks et al. 2014, Handley et al. 2015, Mitchell et al. 2015), as well as in other orders such as Lepidoptera (e.g., Devries et al. 2008, Groot et al. 2016, Liénard and Löfstedt 2016, Silk and Eveleigh 2016). In cases of sympatric species that overlap in both seasonal and diel phenology, the presence of additional components in their pheromones may assist in segregation by augmenting or synergizing responses by conspecifics and/or antagonizing responses by heterospecifics. Segregating effects of minor pheromone components have been reported several times in the Cerambycidae, especially in the subfamilies Cerambycinae and Lamiinae (e.g., Hanks and Millar 2013, Mitchell et al. 2015, Meier et al. 2016, Hanks et al. 2019).

Recently, monochamol was identified in headspace volatile collections of male *M. maculosus* and antennal and behavioral responses of conspecifics to this pheromone have been confirmed in both laboratory and field settings (Andrade et al. 2024). Previous studies indicate that, while analyses of volatile collections of male *M. scutellatus* sampled over 8-h periods presented no detectable differences in quantities of monochamol produced, *M. scutellatus* has been reported to fly earlier in the season and day when compared to *M. notatus* in the northeastern United States (Hanks and Millar 2013, Pimentel et al. 2014, Skabeikis et al. 2016). The objectives of our study were to look for additional minor pheromone components, and differences in diel rhythms of pheromone release and in seasonal and diel flight activity patterns in sympatric populations of *M. scutellatus*, *M. maculosus*, and *M. notatus* as potential mechanisms contributing to the reproductive isolation of these species in northern mixed wood forests of Ontario, Canada. We hypothesized that; (i) if *M. maculosus*, *M. scutellatus,* and *M. notatus* all produce monochamol as their aggregation -sex pheromone, then additional compounds might exist in the volatile collections of males that would minimize the chance of cross-attraction to monochamol, and (ii) differences among the three species in daily and seasonal patterns of pheromone release and/or flight activity might contribute to their reproductive isolation.

Material and Methods

Study Sites

Field trapping experiments were conducted in mixed wood forest stands that had been clear-cut in the Algoma Region, ON, Canada (47°07’56.7‘N 83°08’31.3’W and 47°03’52.0”N 83°09’00.9”W). Stands were primarily composed of *Pinus banksiana* Lamb. (Pinales: Pinaceae) (ca. 80%) harvested within 2 years prior to the experiments using either the short-wood method (cut-to-length) (220.5 ha harvested in 2018, and 55.7 ha harvested in 2020) or full tree harvest method (33.2 ha harvested in 2015, and 18.5 ha harvested in 2016). Stands used for field trials are part of the Timagami Section (L.9—sensu Rowe 1972) of the Great Lakes-St. Lawrence Forest Region in which *Pinus strobus* L., *P. resinosa* Aiton (all Pinales: Pinaceae) and *P. banksiana* are commonly found along with *Populus grandidentata* Michx. (Malpighiales: Salicaceae), *Betula alleghaniensis* Britton, *Ostrya virginiana* (Mill.) K.Koch (all Fagales: Betulaceae), *Acer saccharum* Marshall and *Acer rubrum* L. (all Sapindales: Sapindaceae) (Wester et al. 2018, Ontario Ministry of Natural Resources and Forestry 2020).

Source of Insects

All beetles used for laboratory assays were collected from clear-cut mixed wood forests in the Algoma Region, ON, Canada (47°07’56.7‘N 83°08’31.3’W) following the same methodology as Andrade et al. (2024). In brief, beetles were collected in 12-unit multi-funnel traps (Lindgren 1983) equipped with live collecting cups (ie, dry collecting cups with metal mesh covered holes on the sides and bottom to drain rainwater and catch beetles alive) baited with semiochemicals. A total of 20 traps [treated with Fluon to increase beetle catches (Allison et al. 2011, 2016)] were deployed in four linear arrays, spaced 30 m apart. Each array had five traps 30 m distant from each other (i.e., each array was 120 m long). All traps were baited with ultra-high release (UHR) α-pinene lures (98% purity and release rate of 2,000 mg/day at 25 °C) and monochamol bubblecap lures (99% purity and release rate of 1.5 mg/day at 30 °C) purchased from Synergy Semiochemicals (Delta, British Columbia, Canada). Live collecting cups were half-filled with fresh *P. resinosa* foliage to serve as food for captured beetles. Foliage inside the collecting cups was replaced, and captured beetles collected weekly from June to October in 2020 and 2021. Beetles were transferred to the laboratory, sorted, and identified to sex and species using morphological characters (Bousquet et al. 2017). They were then placed in cages (41.3 cm in length × 40.6 cm in width × 61.6 cm in height) in groups ranging from 30 to 80 individuals of the same species and sex with fresh *P. resinosa* foliage and cotton balls soaked in water. All beetles were kept under controlled conditions at approximately 25 °C and 60% RH under a LD 16:8 h cycle for a minimum of 10 days prior to assay.

Volatile Collection and Pheromone Identification

The presence of additional pheromone compounds was investigated with headspace volatile collections of male and female beetles in the laboratory following the same methodology as Andrade et al. (2024). In summary, groups of 3 males or 3 females of *M. maculosus*, *M. notatus* and *M. scutellatus* were placed in glass aeration chambers (21 cm in length × 6.4 cm outside diameter) separated by sex and species. Beetles were separated in individual compartments by metal mesh septa inside aeration chambers to avoid aggressive interactions between individuals during sampling. Each compartment held one sheet of filter paper (Whatmann nº 1, 5.5 cm diam.) folded in half, making a crease that allowed the beetle to crawl underneath for shelter. One fresh twig of *P. resinosa* (leaves removed) and one cotton ball soaked with distilled water were given to each individual beetle on a weekly basis. Aeration chambers were inspected daily, and any dead beetles were replaced with live ones.

Aerations of groups of male and female beetles were conducted at ~25°C and 60% RH under a LD 16:8 h cycle. Beetles were also exposed to two 60W-LED, full-spectrum, plant growth lights for five hours during the photophase (from 10:30 to 15:30). Charcoal filtered compressed air was continuously drawn in one end of the aeration chamber at 500 ml/min, and volatiles from groups of male and female beetles were trapped in ORBO 1103 Porapak-Q (50/80) columns (150/75 mg; Sigma-Aldrich, St Louis, Missouri, United States) fitted to the other end. Volatiles from each aeration were collected by eluting the Porapak-Q columns with 8 × 200 µl of double-distilled HPLC grade hexane every 24, 48, or 72 h between 5 September and 22 October 2020 and between 14 July and 27 September 2021. Extracts were concentrated to 100 µl per individual (i.e., one insect equivalent per 100 µL) under a gentle stream of nitrogen and stored at −20°C until analysis by gas chromatography mass spectrometry (GC–MS).

Identification of monochamol in the volatile samples was performed by matching the retention time and mass spectrum to those of a monochamol authentic standard solution at 25 ng/ml in hexane on GC–MS. A blend of straight-chain hydrocarbons at 100 μg/ml in cyclohexane (C10—C40, even and odd) was used as reference to calculate the retention index (RI) values for monochamol from volatile samples and monochamol authentic standard solution on GC–MS.

Diel Rhythms in Pheromone Release

The effects of photoperiod on pheromone release by *M. scutellatus* were investigated with volatile collections of males in the laboratory following the same methodology as outlined under Materials and Methods in *Volatile collection and pheromone identification*. Volatile collections of a group of 3 male *M. scutellatus* were performed during the 16 h of photophase (from 06:00 to 21:55) and 8 h of scotophase (from 22:00 to 05:55) for 7 consecutive days (between 9 and 16 August 2021) ($n = 7$ photophase volatile extract samples; 7 scotophase volatile extract samples; 14 volatile extract samples total). Volatile collections were conducted based on observations of natural photophase and scotophase hours during the time of the year which beetles were observed to be most active in the field (between mid-July and mid-August). All extract samples were analyzed by GC–MS and the amount of monochamol (in ng/ml) that the male beetles produced during each period (16-h photophase period and 8-h scotophase period) was calculated and compared to an external standard based on a calibration curve calculated for monochamol at concentrations of 10, 25, 50, 75, and 100 ng/ml in hexane ($R^2 = 0.9978$). It was not possible to investigate the effects of photoperiod on pheromone release by *M. maculosus* due to the low numbers of males available in the lab colony when volatile collections were conducted.

Phenological and Diel Patterns of Flight Activity

Diel patterns of response to the pheromone monochamol and flight phenology of *M. scutellatus*, *M. maculosus*, and *M. notatus* were characterized with 12-unit, multi-funnel traps (Lindgren 1983) (treated with Fluon as in *Source of insects*) baited with UHR α-pinene, UHR ethanol, and monochamol bubblecap lures in 2016, 2017, and 2019. Lures were purchased from Synergy Semochemicals and had a purity and release rate of 98% and 2,000 mg/day at 25 °C for UHR α-pinene, 95% and 250 mg/day at 25 °C for UHR ethanol, and 99% and 1.5 mg/day at 30 °C for monochamol. In total, 2 traps were used in 2016 and 4 traps were used in 2017 and 2019. Traps were deployed in a linear array with 50 m distance between traps and collected until the total number of *Monochamus* captured per week was no higher than one (between 13 July and 13 October 2016; between 14 June and 10 October 2017; and between 28 June and 3 October 2019). In 2016, traps were collected on 13 July, 18 July, and daily between 20 and 22 July, between 25 and 29 July, between 2 and 5 August, and between 8 and 12 August, and weekly between 18 August and 13 October ($n = 27$ dates of collection total). Traps were collected weekly in 2017 ($n = 18$ dates of collection total) and 2019 ($n = 15$ dates of collection total). All traps were placed in clear-cut mixed wood forests in the Algoma Region, ON, Canada (47°07′56.7″N 83°08′31.3″W and 47°03′52.0″N 83°09′00.9″W). All traps used in 2016, 2017, and 2019 had an 8-chamber collection basin installed (BioQuip Products), which was programmed to rotate among chambers every 3 h starting at 00:01. Due to harvesting activity in 2019, the traps had to be removed from the clear-cut site and relocated in a nearby forest on 14 August 2019. Traps placed in the forest did not have the collection bottle rotator and were equipped with a wet collecting cup containing ~200 ml of antifreeze for preservation of captured specimens. During the 3 years of collection, the contents of each cup were sieved, placed in a Whirl-Pak bag (24 oz, Nasco) containing 95% ethanol and transported to the laboratory where they were kept at 5°C until processed. Beetles were sorted and morphological characters were used to sex and identify specimens to species (Bousquet et al., 2017).

Chemical Analyses

All GC–MS analyses were performed on an Agilent 7890B GC with 5977B MS fit with an HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies) operated in EI mode (70eV). The carrier gas was Helium (constant flow rate of 1 ml/min) and the oven temperature was held at 40 °C for 1 min, then programmed to increase at 7 °C/min to 280°C and held for 10 min. Injection was splitless with injector temperature set to 250 °C, transfer line temperature set to 280 °C, ion source temperature set to 230 °C, and MS quadrupole temperature set to 150 °C. A 40–300 m/z mass scanning scope was used on full scan mode at an acquisition rate of 50 Hz. Tentative identification of monochamol present in male volatile extracts was performed by comparing retention indices and mass spectrum to an authentic standard. Peak integration was performed manually with the Agilent MassHunter Data Analysis Software.

Statistical Analyses

Calculations were performed to estimate quantities of monochamol produced per hour by individual male *M. scutellatus* that was recovered in each 16-h photophase and 8-h scotophase extract samples based on volume (each sample had a final volume of 300 μl). The Wilcoxon Signed Rank Test on Paired Samples ($\alpha = 0.05$) was used to compare differences in the median quantities of monochamol produced per hour (ng/h) by individual males during the photophase and scotophase periods.

The mean number of male and female *M. maculosus*, *M. notatus*, and *M. scutellatus* collected per collecting cup (which was rotated every 3 h by the collection bottle rotator) per week was calculated for the period in which there was more than one date of collection within a week (between 18 July and 12 August 2016). Generalized linear models (GLM) with binomial distribution were used to analyze the effect of collection date on the captures of individual *Monochamus* species per trap as a proportion of the total of all *Monochamus* spp. adults collected within weeks in individual years (seven GLM in total; one GLM for each species per year).

Diel patterns of flight activity of individual *Monochamus* species were characterized by combining the data collected over 3 years and calculating the cumulative proportion of *M. maculosus*, *M. notatus*, and *M. scutellatus* captured per trapping time interval. Differences in the distribution of species across time intervals were described using descriptive analysis. All analyses were performed with $R_{4.2.2}$ (The R Foundation for Statistical Computing, 2022).

Results

Volatile Collection and Pheromone Identification

A total of 64 volatile extracts were collected from 3 male and 3 female *M. maculosus* ($n = 32$ volatile samples per sex); 26 volatile extracts were collected from 3 male and 3 female *M. notatus* ($n = 13$ volatile samples per sex); and 28 volatile extracts were collected from 3 male and 3 female *M. scutellatus* ($n = 14$ volatile samples per sex) respectively. Due to the low numbers of *M. notatus* available in the lab colony to replace those that died in the aeration chambers, we continued collecting volatile extracts from remaining beetles until they were all dead. Therefore, an additional 42 volatile samples were collected from 2 male and 2 female *M. notatus* ($n = 17$ samples per sex) and 1 male and 1 female *M. notatus* ($n = 4$ samples per sex).

Detectable quantities of monochamol (RI 1619 on a semi-polar HP-5MS column) were present in 10 out of 14 volatile extracts collected from male *M. scutellatus*. No quantities of monochamol were detected in any of the 14 volatile extracts collected from female *M. scutellatus*. No other qualitative differences were detected between volatile extracts of male and female *M. scutellatus*. Analysis of volatile extracts from male and female *M. notatus* by GC–MS showed no obvious qualitative differences between sexes. No volatile extracts from male and female *M. notatus* contained detectable traces of monochamol. All volatile extract analyses of *M. maculosus* used in this study for comparison with those from *M. scutellatus* were from Andrade et al. (2024). Preliminary analyses of the volatile extracts from male *M. scutellatus* and male *M. maculosus* that contained detectable quantities of monochamol showed no obvious qualitative differences between species that also existed between conspecific males and females (i.e., only males produce and release pheromone) (Fig. 1).

Diel Rhythms in Pheromone Release

Detectable quantities of monochamol were present in all 7 photophase and 7 scotophase volatile collections from male *M. scutellatus*. No significant differences were observed in median quantities of monochamol produced per individual per hour during the photophase and scotophase periods (paired-samples Wilcoxon $V = 13$, $P = 0.94$, effect size $r = 0.06$). The mean (±SE) quantity of monochamol produced by individual males across photophase and scotophase periods was 0.086 ± 0.009 ng/h. Quantities of monochamol produced by individual males during the photophase period ranged from 0.051 to 0.175 ng/h [0.084 ± 0.016 (mean ± SE) ng/h], and quantities of monochamol produced by individual males during the scotophase period ranged from 0.067 to 0.122 ng/h [0.088 ± 0.009 (mean ± SE) ng/h]. No traces of monochamol were detected in the 7 photophase and 7 scotophase volatile collections from female *M. scutellatus*.

Phenological Patterns of Flight Activity

Funnel traps placed in the clear-cut area captured a total of 95 *Monochamus* spp. in 2016, 2,335 in 2017, and 2,685 in 2019 (Table 1). In 2019, funnel traps placed in the forest area captured another 435 *M. maculosus* and 98 *M. scutellatus* ($n = 533$ *Monochamus* spp. collected in the forest). Due to the low number of *M. maculosus* collected in 2016, and *M. notatus* collected in 2019, these species were excluded from analyses in those respective years. In 2019, only the individuals captured in the clear-cut area were used for analysis.

Week of collection had a significant effect on the proportion of the total *M. scutellatus* (GLM, ßdate = −0.614, SE = 0.015, $z = -3.984$, $P < 0.001$), and *M. notatus* (GLM, ßdate = 0.00061, SE = 0.015, $z = 3.984$, $P < 0.001$) collected per trap in 2016 (Fig. 2A), and on the proportion of the total *M. scutellatus* (GLM,

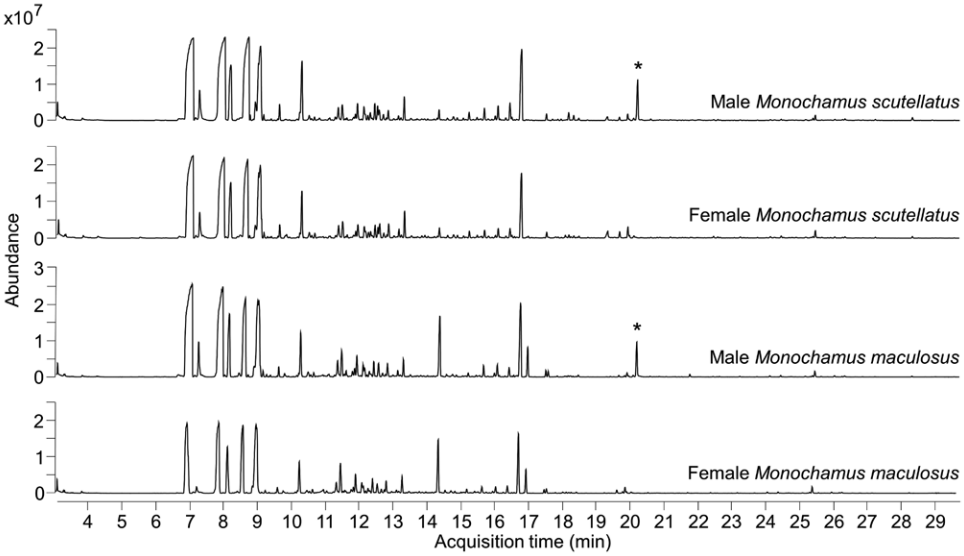


Fig. 1. Total ion chromatogram of 72-h volatile collection from male and female *Monochamus scutellatus* (top), and male and female *M. maculosus* (bottom) on a semi-polar HP5-MS column ($n = 3$ adult beetles each on *Pinus resinosa* twigs). The male-specific compound tentatively identified as monochamol (RI 1619) is indicated by *. Analyses of male and female *M. maculosus* on the bottom-half of the figure were adapted from Andrade et al. (2024) (reproduced with permission from Springer Nature Customer Service Centre, SNCSC).

Table 1. Total number of *Monochamus maculosus, M. notatus,* and *M. scutellatus* collected per week from multi-funnel traps baited with α-pinene, ethanol, and synthetic monochamol during 2016, 2017, and 2019. Collections were performed in clear-cut mixed wood forests in the Algoma Region, ON, Canada

Year of collection	Number of traps used per year	Number of collection dates per year	*M. maculosus*	*M. notatus*	*M. scutellatus*
2016	2	27	8	19	58
2017	4	18	1,076	192	1,067
2019	4	19	599	20	2,066

ßdate = −0.032, SE = 0.001, $z = -16.670$, $P < 0.001$), *M. notatus* (GLM, ßdate = 0.007, SE = 0.003, $z = 2.438$, $P = 0.014$), and *M. maculosus* (GLM, ßdate = 0.029, SE = 0.001, $z = 15.54$, $P < 0.001$) collected per trap in 2017 (Fig. 2B). The majority of *M. scutellatus* were collected earlier in the season compared to *M. notatus* in 2016 (e.g.,≈80% of total *M. scutellatus* trap capture occurred by mid August, while ≈80% of total *M. notatus* trap capture occurred by early September 2016); and to *M. maculosus* and *M. notatus* in 2017 (e.g., ≈80% of total *M. scutellatus* trap capture occurred by mid August, while ≈80% of total *M. maculosus* and *M. notatus* trap capture occurred by early September 2017).

In 2019, similar to the 2017 data, the majority of *M. scutellatus* were collected earlier in the season compared to *M. maculosus* (e.g., ≈80% of total *M. scutellatus* trap capture occurred by late July, while ≈80% of total *M. maculosus* trap capture occurred by mid-August 2019). However, it is unknown whether these proportional differences between the 2 species collected per trap in the clear-cut and forest areas during 2019 are related to time of the year and/or trap location. Week of collection had a significant effect on the proportion of the total *M. scutellatus* (GLM, ßdate = −0.0815, SE = 0.004, $z = -17.66$, $P < 0.001$) and *M. maculosus* (GLM, ßdate = 0.00081, SE = 0.004, $z = 17.66$, $P < 0.001$) collected per trap in the clear-cut area in 2019 (Fig. 2C).

Diel Patterns of Flight Activity

Funnel traps captured a total of 5,115 *Monochamus* spp. during 2016, 2017, and 2019 (Table 2). The majority of *M. scutellatus* were collected earlier in the day compared to *M. maculosus* and *M. notatus* (e.g., ≈80% of total *M. scutellatus* trap captures occurred by 15:01, while ≈80% of total *M. maculosus* and *M. notatus* trap captures occurred by 18:01) (Fig. 3A). However, the peak flight period of these species over 24 h appears to occur at similar intervals in the afternoon (between 12:01 and 15:00 for *M. scutellatus*, and between 15:01 and 18:00 for *M. maculosus* and *M. notatus*) (Fig. 3B).

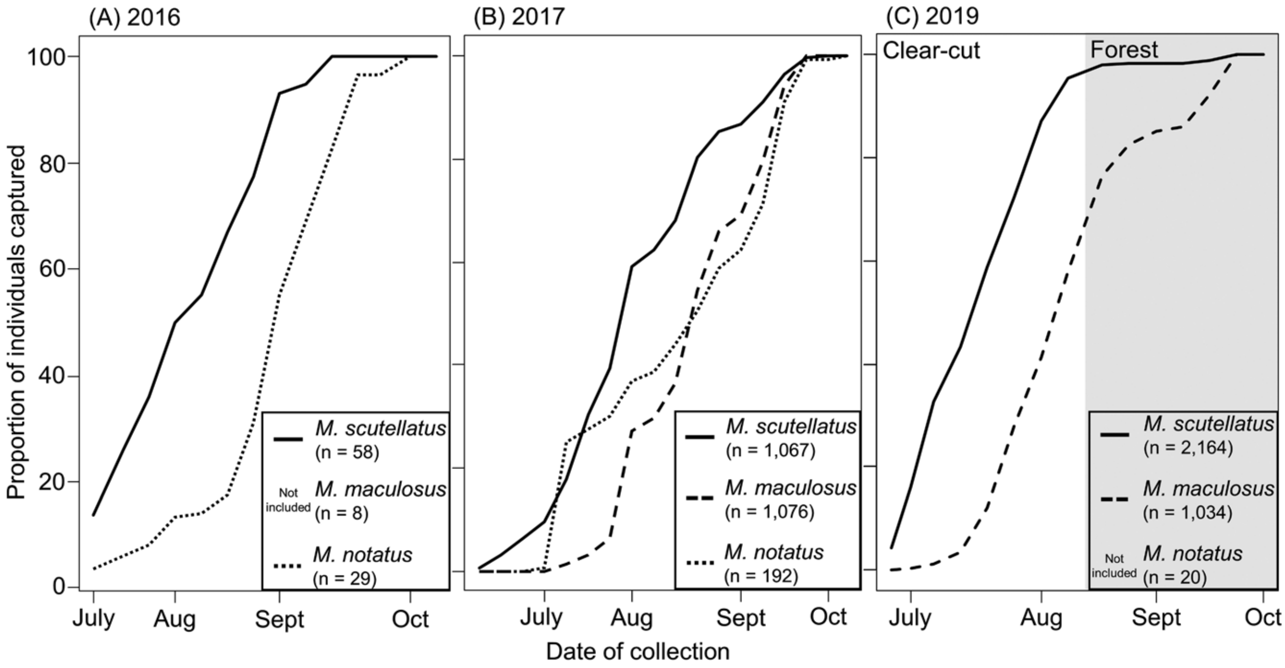


Fig. 2. Cumulative proportion of individual *Monochamus* species collected from multi-funnel traps baited with α-pinene, ethanol, and synthetic monochamol during 2016 (A), 2017 (B), and 2019 (C). Due to the low numbers of individuals collected, *M. maculosus* was not considered for analysis in 2016 and *M. notatus* was not considered for analysis in 2019. Collections performed in the clear-cut and forest areas in 2019 are indicated in (C). Traps were located in clear-cut mixed wood forests in the Algoma Region, ON, Canada, during 2016, 2017, and part of 2019`s collections. The forest area used in 2019 was dominated by *Pinus banksiana*.

Table 2. Total number of *Monochamus maculosus, M. notatus* and *M. scutellatus* collected per week from multi-funnel traps baited with α-pinene, ethanol, and synthetic monochamol and equipped with eight collecting cups that rotated among cups every 3 hours during 2016 (*n* = 27 dates of collection total, 2 traps used total), 2017 (*n* = 18 dates of collection total, 4 traps used total), and 2019 (*n* = 19 dates of collection total, 4 traps used total). Collections were performed in clear-cut mixed wood forests in the Algoma Region, ON, Canada.

Time interval (hours)	*M. maculosus*	*M. notatus*	*M. scutellatus*
00:01–03:00	35	6	5
03:01–06:00	9	0	2
06:01–09:00	7	0	33
09:01–12:00	68	8	288
12:01–15:00	277	63	1,124
15:01–18:00	480	77	1,065
18:01–21:00	345	30	526
21:01–00:00	462	57	148

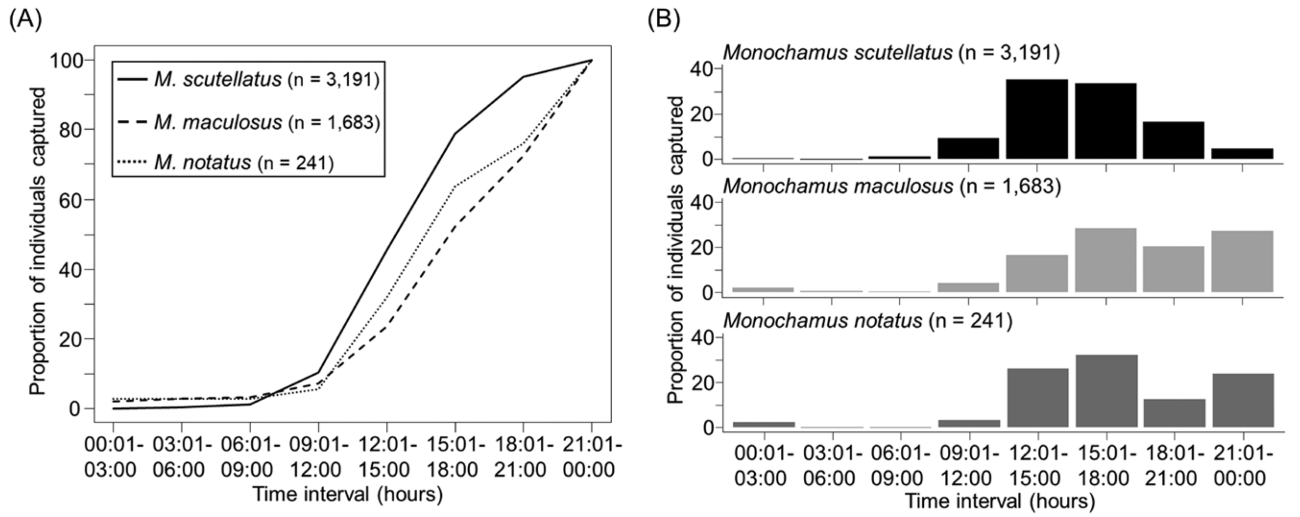


Fig. 3. Cumulative (A) and relative (B) proportion of *Monochamus scutellatus, M. maculosus*, and *M. notatus* collected from multi-funnel traps baited with α-pinene, ethanol, and synthetic monochamol and equipped with 8 collecting cups that rotated among cups every 3 hours during 2016, 2017, and 2019. Collections were performed in clear-cut mixed wood forests in the Algoma Region, ON, Canada.

Discussion

Pheromones mediate mate location and recognition in insects and are generally thought to vary in relative amounts among species, particularly sympatric species (Symonds and Elgar 2008, Wyatt 2014, Allison and Cardé 2016). Pheromone parsimony and absence of additional minor compounds seem common in the genus *Monochamus* since monochamol has been reported to be the aggregation-sex pheromone or a putative pheromone attractant for several species, including the sympatric *M. maculosus*, *M. notatus*, and *M. scutellatus* in eastern Canada (Hanks and Millar 2016, Millar and Hanks 2017, Andrade et al. 2024). Although hybridization between sympatric *Monochamus* species in the field appears to be rare (pers. comm. in Hughes and Hughes 1987), evidence of additional pre-mating mechanisms that facilitate reproductive isolation among *M. maculosus*, *M. notatus*, and *M. scutellatus* is lacking. We found no evidence of additional pheromone compounds in *M. maculosus* and *M. scutellatus* volatile collections that could reduce cross-attraction, but our results from field trapping experiments do provide evidence that differences in diel and seasonal flight activity between these 2 species in response to monochamol may reduce the potential for heterospecific interactions.

Males of *M. scutellatus* and *M. maculosus* were observed to produce monochamol in the laboratory (Andrade et al. 2024, this study). Our results indicate that specific additional minor pheromone components are unlikely to contribute to their reproductive isolation since no obvious qualitative differences between heterospecific males that also existed between conspecific males and females were found in preliminary analyses of volatile collections. In addition, our results show that males of *M. scutellatus* produced equivalent amounts of monochamol during both the photophase and scotophase periods suggesting that diel rhythms associated with pheromone production are unlikely to contribute to reproductive isolation. This result corroborates previous findings of Skabeikis et al. (2016) in which volatile collections from male *M. scutellatus* under natural light exposure sampled in 8 h time intervals observed equivalent amounts of monochamol among samples. Although no evidence of pheromone blend differences between *M. maculosus* and *M. scutellatus* have been found (Andrade et al. 2024, this study), qualitative and quantitative differences in sex pheromones can serve as an important pre-mating isolating mechanism for sympatric species that share similar pheromones in the Lepidoptera (Eizaguirre et al. 2009, Yang et al. 2009, Allison and Cardé 2016, Chen et al. 2018) and Cerambycidae (Hanks and Millar 2013, Hanks et al. 2019).

We observed that *M. scutellatus* emergence begins earlier in the season than the other two sympatric *Monochamus* spp. suggesting some seasonal separation between them. Interactions between *M. scutellatus* and *M. notatus* in the field seem uncommon (Hughes and Hughes 1987) and we hypothesize that this could be due to seasonal separation since it is known to occur in the United States (Hanks and Millar 2013, Pimentel et al. 2014). Temporal segregation among species that use the same resource in a similar way and/or the same pheromone has been observed to occur in Coleoptera (Feer and Pincebourde 2005, Hanks et al. 2014, 2019, Mitchell et al. 2015), Diptera (Sladecek et al. 2017), and Lepidoptera (Greenfield and Karandinos 1979, Devries et al. 2008). While no reports of interspecific interactions between *M. maculosus* and *M. notatus* were found in the literature, our results show considerable overlap in the flight phenology of these 2 species. This result suggests a higher potential for interspecific interactions between *M. maculosus* and *M. notatus* in the field considering that both species use *Pinus* spp. as host plants (Bousquet et al. 2017) and have demonstrated strong attraction to monochamol in field trials (Fierke et al. 2012, Ryall et al. 2015). In addition, our results from *M. notatus* volatile and field trapping collections are consistent with a previous report that *M. notatus* may not produce monochamol but could use the pheromone produced by sympatric *Monochamus* spp. as a kairomone to locate suitable hosts and mating sites (Skabeikis et al. 2016).The observed differences in diel flight patterns between *M. scutellatus*, *M. maculosus*, and *M. notatus* (*M. scutellatus* was observed to fly earlier in the day than *M. maculosus* and *M. notatus*) likely also contribute to reproductive isolation of these species. However, this difference in diel flight patterns does not seem strong enough to drive segregation on its own considering that all 3 species peak during similar intervals in the afternoon. Clear diel segregation between *M. notatus* and *M. scutellatus* has been previously reported during late spring, although diel flight activity of *M. notatus* was observed to vary throughout the flight season (Skabeikis et al. 2016). Observational studies have reported that mating activity in *M. scutellatus* occurs during the afternoon

(Hughes 1979, Allison and Borden 2001) and may overlap with *M. notatus* activity (Hughes and Hughes 1987).

Differences in seasonal and diel flight activity among *M. scutellatus*, *M. maculosus,* and *M. notatus* in response to monochamol observed in this study may reduce the potential for interspecific interactions between *M. scutellatus* and the other 2 species. However, temporal segregation does not seem strong enough to prevent cross-attraction between *M. maculosus* and *M. notatus* since our results show considerable overlap in both flight phenology and diel flight activity patterns. Other factors that could facilitate mate recognition at short-range and reduce cross-attraction between sympatric congeners in the Cerambycidae should be considered in the future, including differences in cuticular hydrocarbons and particular host/habitat preferences within forests. Several studies have demonstrated that contact pheromones play an important role in male-mate recognition in *Monochamus* spp. (Hughes 1979, Kim et al. 2006, Ibeas et al. 2009, Brodie et al. 2012), and host plant volatiles have a synergistic or additive effect in the attraction to monochamol in some species in the field (Allison et al. 2012, Fierke et al. 2012, Macias-Samano et al. 2012, Ryall et al. 2015). Differences in abundance among sympatric *Monochamus* spp. across the vertical strata of the forest canopy have been reported (Graham et al. 2012, Dodds 2014), and could also be related to resource partitioning (Paine et al. 1981, Ayres et al. 2001, Mitchell et al. 2015, but see Dearborn et al. 2016) and contribute to reproductive isolation in these species. In addition, visual cues on the elytra may contribute to differentiation between *Monochamus* spp. since visual stimuli (e.g., color, size, and shape) have been reported to have effects at close range in Cerambycidae (Fukaya and Honda 1996, Yasui 2009).

Although we were able to sample the effluvia of many male *M. scutellatus* and *M. maculosus* and validate monochamol as a pheromone component of these two species, inducing calling behavior from *Monochamus* spp. in the laboratory is challenging and limited our ability to collect extracts containing pheromone. We do not exclude the possibility that additional minor pheromone components may exist but were not detected in our volatile extractions. Using live males as baits to collect beetles in future field trapping experiments would be an interesting way to look for evidence of minor species-specific pheromone components.

Our study provides evidence that seasonal and diel separation in flight behavior may be a mechanism that reduces interspecific interactions of *M. scutellatus* with both *M. maculosus* and *M. notatus* and contributes to their reproductive isolation. We found no evidence for additional species-specific minor pheromone compounds and diel rhythms in pheromone production by *M. scutellatus*. However, an increasing body of evidence indicates that differences in contact pheromones and differences in distribution across the vertical strata of the forest canopy could contribute to the reproductive isolation of *M. scutellatus, M. maculosus*, and *M. notatus* and require further investigation.

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Author contributions

Samara Andrade (Conceptualization [Equal], Formal analysis [Lead], Funding acquisition [Supporting], Investigation [Lead], Visualization [Lead], Writing—original draft [Lead], Writing—review & editing [Equal]), Michael McTavish (Formal analysis [Supporting], Visualization [Supporting], Writing—review & editing [Supporting]), Sandy M Smith (Project administration [Supporting], Resources [Supporting], Supervision [Supporting], Validation [Equal], Writing—original draft [Supporting], Writing—review & editing [Equal]), and Jeremy Allison (Conceptualization [Equal], Funding acquisition [Lead], Project administration [Lead], Resources [Lead], Supervision [Lead], Validation [Equal], Writing—original draft [Supporting], Writing—review & editing [Equal])

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Data Availability

The data that supports the findings of this study are available on the Open Government Portal (https://doi.org/10.23687/aaf61ccc-e529-4c60-bb8b-cc76da3ff35a) and will be made available upon publication.

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