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Common Carp Implanted with Prostaglandin $F_{2\alpha}$ Release a Sex Pheromone Complex that Attracts Conspecific Males in Both the Laboratory and Field

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Abstract When ovulated, female fish of many species are known to release a F-prostaglandin-derived sex pheromone that attracts conspecific males. Recently, this pheromone was identified in the common carp as a mixture of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and unidentified body metabolites, which we termed a 'pheromone complex.' The present study sought to test the activity of this pheromone complex in the field by developing a system using carps implanted with $PGF_{2\alpha}$ as pheromone donors. An initial experiment determined that osmotic pumps that delivered up to 0.4 mg of $PGF_{2\alpha}$ per hour could be implanted into carp without any apparent effects on their health. A second experiment found that $PGF_{2\alpha}$-implanted male and female carp released biologically relevant (and equivalent) quantities of $PGF_{2\alpha}$, along with two of its seemingly inactive metabolites, for up to 2 weeks. Laboratory experiments demonstrated that the odor of $PGF_{2\alpha}$-implanted carp was highly attractive to male conspecifics, and included necessary body metabolites; it attracted males as strongly as ovulated carp odor, and much better than $PGF_{2\alpha}$ alone. Finally, a field test demonstrated that $PGF_{2\alpha}$-implanted female carp attracted mature male, but not female carp, from a distance of 20 m. This is the first demonstration of the activity of a $PGF_{2\alpha}$-based pheromone in a natural environment and confirms the use of a PGF-pheromone complex in the carp. We suggest that the implant technique may be useful in future studies of how PGF pheromones function and could be further developed to attract invasive fish for use in control.

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Introduction

Female fish of many species release a species-specific F prostaglandin (PGF)-based sex pheromone when ovulated that attracts males and mediates spawning interactions (Stacey and Sorensen, 2009; Lim and Sorensen, 2011). However, with the exception of the common carp, *Cyprinus carpio* (Lim and Sorensen, 2011), studies of this pheromone have been restricted to the laboratory, and have used synthesized PGFs. Our understanding of the identity and biological function of this class of pheromone is incomplete. Recently, we discovered that the pheromone released by ovulated female common carp (hereafter termed 'carp') is comprised of a mixture of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and unidentified polar body metabolites, which we termed a 'pheromone complex' (Lim and Sorensen, 2011). This discovery suggested that we might be able to induce carp to produce the complete pheromone complex by implanting them with $PGF_{2\alpha}$, so that they would release various PGFs and body metabolites, thereby permitting larger scale field tests. We were especially interested in these tests because there have been very few tests of fish pheromones in the field (Johnson and Li, 2010), and because the carp is a highly invasive species for which few control options exist (Weber and Brown, 2009; Sorensen and Bajer, 2011).

The possibility that $PGF_{2\alpha}$ implants might elicit normal pheromone release is based on several studies of the closely related goldfish, *Carassis auratus*, which, if injected with $PGF_{2\alpha}$ releases a chemical cue(s) for approximately 2 hrs,

which mimics both the behavioral activity and olfactory potency of the natural pheromone (Sorensen et al., 1986, 1988; Sorensen and Goetz, 1993). However, little is understood about $PGF_{2\alpha}$ metabolism and clearance in fish. There appears to have been only one study of the metabolism of exogenously administered $PGF_{2\alpha}$ in fish, and it shows (with a single radiochromatogram) that goldfish injected with radio-labeled $PGF_{2\alpha}$ likely release at least five PGF metabolites, in addition to $PGF_{2\alpha}$ (Sorensen and Goetz, 1993). We do not know whether carp would release a similar suite of PGFs and other metabolites. Effects of gender on $PGF_{2\alpha}$ metabolism in fish are also unknown. Additionally, while osmotic pumps have been used to deliver thyroid inhibitors and steroidal hormones in fish (e.g., Comeau and Campana, 2003; Metz et al., 2003), they have not been tested previously for use with $PGF_{2\alpha}$. There is also the possibility that long-term administration of $PGF_{2\alpha}$ could be toxic to fish, as it is to mammals (Clayman, 1975; Adams, 2001; Lust et al., 2011).

The aim of the present study was to investigate the effects of long-term, constant, inter-peritoneal delivery of $PGF_{2\alpha}$ on pheromone production/release in carp and then, if successful, conduct preliminary field tests. We asked: 1) Is chronically implanted $PGF_{2\alpha}$ toxic to carp? 2) What levels and types of PGFs do implanted male and female carp release, and how do these compare with PGFs released by naturally ovulated carp? 3) Is the odor of $PGF_{2\alpha}$-implanted carp attractive to carp in the laboratory, and how does it compare to ovulated females and/or $PGF_{2\alpha}$alone (i.e., is the complex released)? 4) Does the odor of $PGF_{2\alpha}$-implanted carp attract male and/or female carp in the field?

Methods and Materials

Experimental Fish Juvenile carp were obtained from a fish farm (Osage Catfisheries, Osage Beach, MO, USA) and raised to maturity in 1,000 l, flow-through, circular tanks supplied with 20°C well water at a rate of 10 l/min. Fish were fed a mixture of flake fish food (Aquatic Ecosystems, Apopka, FL, USA) and frozen brine shrimp (San Francisco Bay Brand, Newark, CA, USA) each day, while being maintained on a 16:8 h (Light: Dark) photoperiod. Once mature (3 yr of age, 500–1,000 g weight), they were sorted by sex (fish were stripped to determine whether they were spermiating or rotund with eggs; i.e., females were not ovulatory), and placed in separate flow-through tanks until needed.

All protocols described in this manuscript were approved by the University of Minnesota Institutional Animal Use and Care Committee.

Osmotic Pheromone Pumps Commercially available osmotic pumps (model 2ML1; Alzet, Durect Co., Cupertino, CA, USA) were selected, based on their use in mammals. The desired quantity of $PGF_{2\alpha}$ (Cayman Chemical, Ann Arbor, MI, USA; see below) was first dissolved in deionized, sterile water, and then injected into the reservoirs of sterile pumps with a syringe. Healthy adult carps were anesthetized by first placing them into an aerated solution of 0.02% MS-222 (Western Chemical Inc., Ferndale, WA, USA), and then into a wet foam cradle. Several scales then were removed from a small area, ventro-posterior to their pelvic fins, and a 4 cm incision made into the body cavity, using a sterile #11 scalpel, through which $PGF_{2\alpha}$-loaded osmotic pumps were inserted. Four stitches, with absorbable monofilament suture (PDS-II, Ethicon, MI, USA), were used to close the incisions. Finally, fish were removed to a 100 l recovery tank and, once upright and mobile, returned to their holding tanks. This operation took only a few minutes and elicited little bleeding and no infection. Additional technical details on the operation and pumps can be found in a technical manual (Lim and Sorensen, 2010).

Question 1. Is $PGF_{2\alpha}$ Toxic to Carp when Chronically Administered by an Osmotic Pump? Our first experiment sought to determine how much $PGF_{2\alpha}$ we could safely administer to carp. Six doses of $PGF_{2\alpha}$ were selected, based on the quantity of $PGF_{2\alpha}$ we previously injected into goldfish [1 $mg \cdot kg^{-1}$; Sorensen et al. (1988)] and on published delivery rates of osmotic pumps into the body cavity. These doses were: 0.0 g $PGF_{2\alpha}$/kg body weight (blank control), 0.04 g/kg (a dose that delivers 0.1 mg/h into the body cavity), 0.2 g/kg (0.5 mg/h), 0.4 g/kg (1.0 mg/h), 1.2 g/kg (3.0 mg/h), and 2.0 g/kg body weight (5.0 mg/h). Three immature female carp [1,020±39 g; gonadosomatic index (GSI: gonad weight/whole body weight x 100%) of 11±2%] were implanted with each dose and then placed into a single large holding tank (1,000 l) that received aerated, 20°C well water. Fish feeding and swimming behavior was observed twice a day, and fish observed not to be feeding or actively moving (i.e., dead or moribund) were removed and euthanized in 0.1% MS-222. The experiment ended after 2 wk when the implants had delivered all of their contents.

Question 2. What is the Fate of the $PGF_{2\alpha}$ Implanted into Male and Female Carp? Having determined the highest dose that a 1 kg carp can tolerate is 0.4 g of $PGF_{2\alpha}$, we next sought to determine how much $PGF_{2\alpha}$ these fish were releasing into the water, and whether gender had an influence on PGF metabolism and release. All three PGFs [$PGF_{2\alpha}$, 15-keto-$PGF_{2\alpha}$ (15 K-$PGF_{2\alpha}$), and 13,14-dihydro-15keto-$PGF_{2\alpha}$ (dh15K-$PGF_{2\alpha}$)] known to be released by naturally ovulated carp were monitored (Lim and Sorensen, 2011). Three males (876±23 g; GSI=8.5±1.1%) and three females (903±20 g; GSI=10.5±2.6%) were implanted with osmotic pumps containing 0.4 g $PGF_{2\alpha}$, while three others of each sex were implanted with pumps containing

water only, as controls. All fish then were placed into 1,000 l holding tanks supplied with 20°C well water. Implanted fish were fed daily for 18 d while being removed to individual 50 l holding tanks 1, 5, 9, 13, 18 d after implantation, for 1 h, and then returned. One liter water samples were collected from these holding tanks and then either used immediately in behavioral tests (see experiment 3, below) or extracted for analysis, by passing them through activated C18 Sep-Pak cartridges (Waters, MA, USA) at a rate of 1 liter/h and eluted with 5-ml of methanol, following established protocols (Lim and Sorensen, 2011).

For analysis, the eluant from extracted holding tank water was dried under a stream of nitrogen, reconstituted in 1 ml of methanol/distilled water (50:50 v/v), and then analyzed by LC-MS/MS (Liquid chromatography-tandem mass-spectrometry) by injection (and standards) onto a LC-MS device equipped with an Eclipse XDB C18-RS column (250×4.6 mm, particle size 5 μm; Agilent Technologies, MA, USA). To separate PGFs, we used a mobile phase of 0.1% formic acid in acetonitrile and 0.1% formic acid in distilled water, at a flow rate of 1 ml/min. The solvent gradient changed from 35% acetonitrile at 0 min to 65% at 12 min (this gradient separated all three PGFs by over 1 min). The LC was interfaced with an ion-trap mass spectrometer (LCQ-1, ThermoScientific, MA, USA) with an electrospray ionization source [ion trap operated in the negative ion mode, with a spray voltage of 5 kV; sheath gas was 99% pure nitrogen at 60 psi; sheath fluid was 50:50 20 mM triethylamine: acetonitrile (v/v)]. We quantified $PGF_{2\alpha}$, 15K-$PGF_{2\alpha}$, and dh15K-$PGF_{2\alpha}$ by summing all peaks associated with each compound's daughter ions, and then extrapolating these values to a five-point calibration curve, which we had previously created by adding standards to immature carp holding water, extracting it, and measuring its contents as described above. This technique controls for both extraction efficiencies and matrix effects; both its accuracy and precision have been shown to be high (Fine et al., 2006; Lim and Sorensen, 2011). Our calibration curves were linear (R^2 values of 0.92, 0.96, and 0.96) and had 95% confidence intervals that ranged from 23–28% around the mean. Mean release values of males and females (across days) were compared for each PGF using two-way repeated measure ANOVAs (normality was initially confirmed using a Kolmogorov-Smirnov test) (SigmaStat, Ashburn, VA, USA). If significance ($P<0.05$) was indicated, daily values were compared to each other using a Holm-Sidek adjustment (Aickin and Gensler, 1996).

Question 3. Is the Odor of $PGF_{2\alpha}$-implanted Carp Attractive and, if so, How Does it Compare with the Natural Female Pheromone and $PGF_{2\alpha}$ Alone (Is a Complex Released)? Having demonstrated that carp implanted with $PGF_{2\alpha}$ release considerable quantities of $PGF_{2\alpha}$, our next step was to determine if the odor of $PGF_{2\alpha}$-implanted carp was pheromonally active (attractive) and, if so, how it compared with the natural pheromone? Two sets of experiments, using an established 2-choice behavioral assay (Lim and Sorensen, 2011; below), addressed these questions. Our first set of experiments tested the attractiveness of $PGF_{2\alpha}$-implanted male and $PGF_{2\alpha}$-implanted female holding water as well as $PGF_{2\alpha}$ against blank water. $PGF_{2\alpha}$-implanted male and $PGF_{2\alpha}$-implanted female holding waters were each tested at three concentrations (full strength, diluted 10,000 times, and diluted 100,000 times; dilutions were performed prior to addition to the mazes), using samples collected from female carp (as part of question 2), when $PGF_{2\alpha}$ release rates peaked (days 5–9). Their holding waters contained $0.7\pm0.2\times10^{-8}$ M $PGF_{2\alpha}$. Blank water control, the water of blank-implanted carp, and samples of previously collected and thawed, ovulated female carp holding waters (previously estimated to contain $2\pm0.3\times10^{-10}$ M $PGF_{2\alpha}$; Lim and Sorensen, 2011), also were tested. Finally, for comparison, a third set of experiments tested the attractiveness of $PGF_{2\alpha}$ alone at concentrations which included those found in $PGF_{2\alpha}$-implanted fish water (i.e. 10^{-11} M–10^{-7} M). Responses (relative attraction, see below) within each series were evaluated by one-way ANOVA (Sigmastat, VA, USA) and, if significant ($P<0.05$), responses to individual odors were compared to each other and blank well water, by pair-wise comparisons using a Holm-Sidek adjustment (Aickin and Gensler, 1996).

The second set of experiments sought to determine the precise potency of $PGF_{2\alpha}$-implanted carp odor relative to whole pheromone (ovulated female odor), as well as $PGF_{2\alpha}$ alone, using head-to-head tests. It used the same two-choice behavioral assay as the first set of experiments, but substituted a test odor for a control. Holding water from $PGF_{2\alpha}$-implanted female carp ($0.7\pm0.2\times10^{-8}$ M $PGF_{2\alpha}$) was tested directly against ovulated female holding water (2×10^{-10} M $PGF_{2\alpha}$). Next, we tested $PGF_{2\alpha}$-implanted female fish holding water against 10^{-7} M $PGF_{2\alpha}$ (the concentration the holding water contained), and then against a concentration of $PGF_{2\alpha}$ (5×10^{-7} M) that was five times greater. As a final follow-up test, we tested 10^{-7} M $PGF_{2\alpha}$ vs. 5×10^{-7} M $PGF_{2\alpha}$, to determine what role $PGF_{2\alpha}$ concentration alone might have had in the previous experiment. Each experiment was analyzed using a one-sample *t*-test (MiniTab, PA, USA), after confirming that data were normally distributed (Kolmogorov-Smirnov test; see Lim and Sorensen, 2011).

Both sets of experiments used a well-established laboratory assay, which used groups of male carp that had previously been activated ('primed') by exposing them to a pre-ovulatory steroid pheromone 8 h prior to experiments (10^{-10} M 17α,20β-dihydroxy-4-pregnen-3-one; Lim and Sorensen, 2011). Each experiment had 10 trials, each of which used a different group of 5 fish. Briefly, for each

trial, groups of males were placed into circular two-choice (1.5 m diameter) mazes, supplied with well water and equipped with overhead cameras and infrared lighting. The distribution of all carps was noted at 15 sec intervals, during a 15 min pre-test period, after which a test odor was added to one end, and either a control (well water alone) or a matching test odor (for head-to-head tests) to the other, using pumps (10 ml/min). Changes in the percent time spent in the test area were calculated to yield a relative attraction value (%). Fish were re-used after a month-long interval in their holdings tanks if, and as, necessary.

Question 4. Is the Odor of $PGF_{2\alpha}$-implanted Carp Attractive to Male and/or Female Carp in the Field? Finally, the attractive properties of implanted-carp water were tested in Lake Keller (Maplewood, MN, USA; 45°00′11.80″N, 93°03′45.48″W). This lake was thought to be representative of many in the region. It is shallow (3–4 m deep), has a surface area of 29 ha, is connected to two other lakes by small streams, and contains a large population of native fish, including bluegill sunfish, *Lepomis macrochirus*, as well as dense beds of submersed vegetation. At the time of the study, Lake Keller was also known to have had approximately 1,500±200 adult carp from mark-recapture studies (Bajer et al., 2011; Chizinski and Sorensen, unpublished results). We used radio-tagged fish to monitor the proximity of male and female carp relative to $PGF_{2\alpha}$-implanted females that were placed into one of two screened (darkened) traps. We chose not to use blank-implanted fish as controls, because laboratory experiments showed they have no influence on male behavior (Lim and Sorensen, 2011; this study), and the lake was already filled with spent females.

The experiment started in early May 2009 (prior to carp-spawning season), when we captured 20 adult carp from around the entire perimeter of the lake (12 spermiating males and 8 mature, but non-ovulated, females) using boat-electrofishing, implanted them with radio-tags (F1850, Advanced Telemetry Systems, MN, USA) following established procedures (Bajer and Sorensen, 2010), and then released them where they were caught. We then monitored the lake for spawning activity on a daily basis (carp spawn in floating vegetation and are easy to spot from shore). At the conclusion of the spawning season in mid-June (i.e., when males were likely to be still searching for spawning females, although none would have been present), we captured two spent female carp (2.96 and 2.65 kg) by boat-electrofishing, implanted them with osmotic pumps containing 900 mg of $PGF_{2\alpha}$ (ca. 0. 4 g/kg of fish), and placed them into one of two wood-framed, mesh-covered traps (1.2×1.2×2.4 m in W×H×L; mesh size=5×5 cm; V-shaped funnel opening 50 cm tall and 5 cm wide), which we placed in an area where spawning had been observed (depth=1.2 m), at a distance of 100 m from each other. A battery-powered bilge pump (4,500 l/h; T1200 Tsunami Bilge Pump, Attwood Marine Products, Lowell, MI, USA) was placed in the back of each trap to create a plume that we estimated, from dye (Bright Dyes, Kingscote Chemicals, Miamisburg, OH, USA) tests, to extend to about 10 m. We then spent 1.5–2.5 h each morning locating and bi-angulating the positions of radio-tagged carp near each trap, using shore-based receivers (estimated precision of 10 m). We focused on regions located 20 m around each trap, as these would have included the pheromone plume. The pair of implanted carp was moved between the two traps every 3 d, so that the position of the control and pheromone traps varied in a way to control for trap location. The experiment continued for 12 d, after which the implants were designed to run out. For analysis, the locations of male and female radio-tagged individuals were determined (LOAS 4.0; Ecological Software Solutions, Sacramento, CA, USA) and analyzed using ArcGIS (ESRI; Redlands, CA, USA). We calculated the number of radio-tagged male and female carp within 20 m of the test and control traps, and compared each by paired *t*-tests (Minitab, PA, USA). Because we were tracking a population of carp that was free to come and go across the lake (and data showed that they did), assumptions of statistical independence were met.

Results

All carp implanted with 0.0, 0.04, 0.1, 0.2, and 0.4 g $PGF_{2\alpha}$ survived, while all those implanted with either 1.2 or 2.0 g $PGF_{2\alpha}$/kg body weight died or were moribund (and sacrificed) within 4 days (Fig. 1). Carp that died fed little, did not move actively, and had skin lesions.

Both implanted male and female carp released very high and similar levels of $PGF_{2\alpha}$ to the water (about 1 mg/h after day 5), while 15K-$PGF_{2\alpha}$ and dh15K-$PGF_{2\alpha}$ were released in far smaller quantities (about 1,000 fold less); the latter did not differ between the sexes (Fig. 2: $P>0.05$). Release rates of all three PGFs peaked at day 5, and declined after 9 days

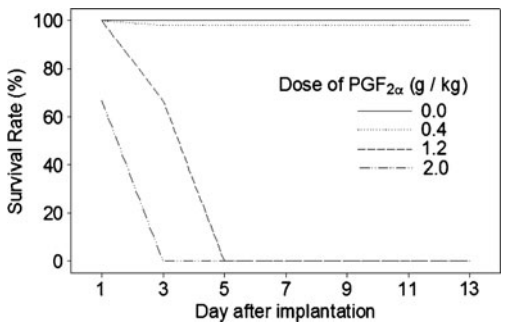

Fig. 1 Survival rates of female common carp implanted with four representative doses of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; g $PGF_{2\alpha}$/kg body weight). All carp implanted with 0.04, 0.1, and 0.2 g $PGF_{2\alpha}$/kg survived, but these data are not shown (to simplify the figure)

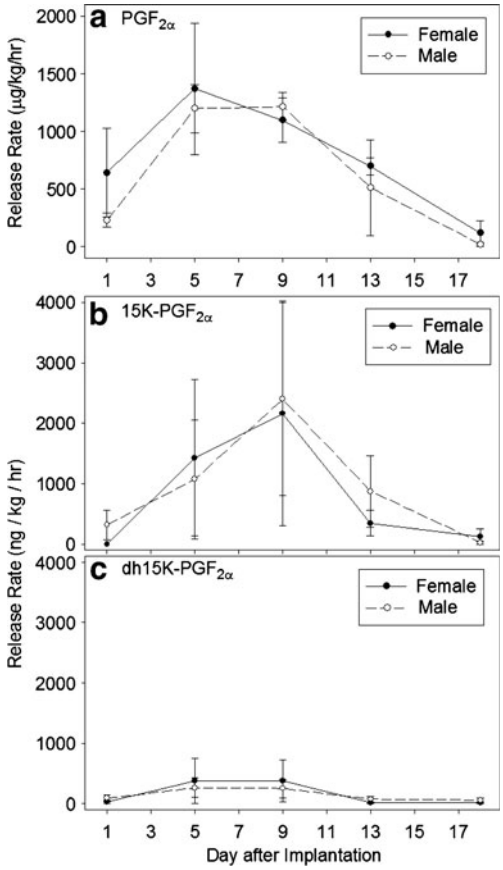

Fig. 2 Release rates (mean ± SEM) of three F prostaglandins a) prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), b) 15-keto- prostaglandin $F_{2\alpha}$ (15 K-$PGF_{2\alpha}$), and 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (dh15K-$PGF_{2\alpha}$), over a 2 wk period, by female (solid circles) and male common carp (empty circles) implanted with 0.4 g/kg body weight of $PGF_{2\alpha}$

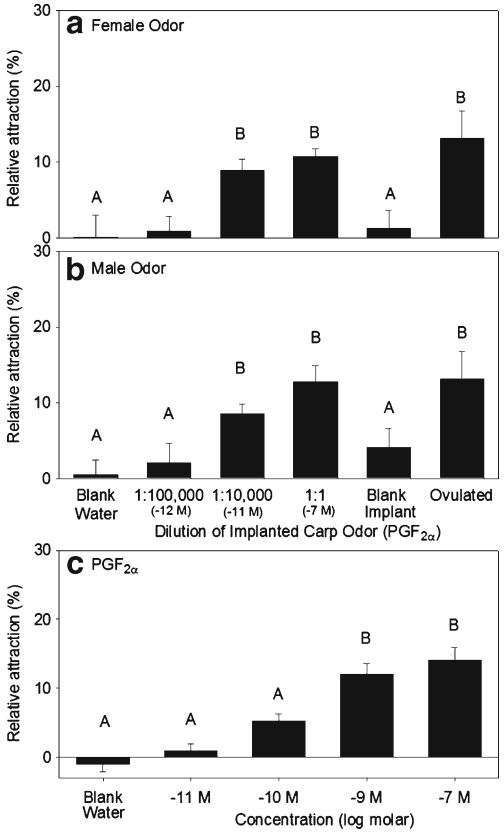

Fig. 3 Attraction (mean relative attraction ± SEM) of primed male common carp to: a) increasing dilutions of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-implanted, blank-implanted, and naturally ovulated female carp odor; b) increasing dilutions of $PGF_{2\alpha}$-implanted, blank-implanted male carp, and naturally ovulated female carp odor; c) different concentrations of $PGF_{2\alpha}$ (log molar). The estimated concentrations of $PGF_{2\alpha}$ in each diluted odor are given in parentheses. Reponses were compared by one-way ANOVA. Different letters indicate differences, $P<0.01$

(Fig. 2: $P<0.05$). The average release rates of all three PGFs could, when summed, account for all of the $PGF_{2\alpha}$ being delivered by the pumps.

While the holding water of blank-implanted female and male carp did not attract males (Fig. 3a, b: $P>0.05$), both $PGF_{2\alpha}$-implanted male, and $PGF_{2\alpha}$-implanted female carp-holding waters attracted male carp in laboratory mazes, even after being diluted 10,000 times (Fig. 3a, b: $P<0.01$). The concentration of $PGF_{2\alpha}$ in the 10,000x diluted water was estimated to be 10^{-11} M (Fig. 3a, b). The level of relative attraction exhibited to both implanted male and implanted female carp holding water was equivalent in magnitude to that of ovulated female water (Fig. 3a, b: $P>0.05$). Maze tests of $PGF_{2\alpha}$ alone showed that while it was attractive at 10^{-9} M (Fig. 3c: $P<0.05$), only weak, non-significant attraction was observed at 10^{-10} M (Fig. 3c). Follow-up, head-to-head tests demonstrated that implanted-carp odor was as potent as ovulated-carp odor, and that implanted odor was far more attractive than $PGF_{2\alpha}$ alone, at both 10^{-7} M (equimolar) and 5×10^{-7} M (Fig. 4). Nevertheless, male carp preferred a concentration of 5×10^{-7} M $PGF_{2\alpha}$ to that of 10^{-7} M $PGF_{2\alpha}$ (9.5±1.3% relative attraction; $P<0.01$).

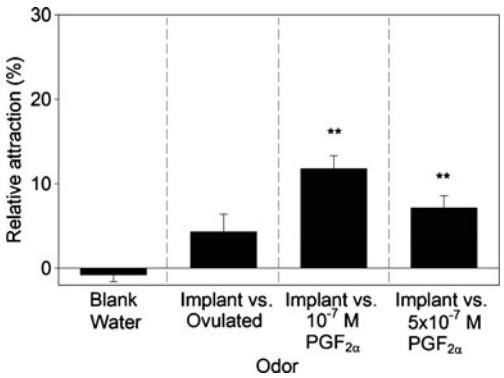

Fig. 4 Attraction (mean relative attraction ± SEM) of primed male common carp to well water vs. well water control, the whole odors from implanted female vs. naturally ovulated carp, the odor of implanted female carp vs. an equimolar concentration of $PGF_{2\alpha}$ (10^{-7} M $PGF_{2\alpha}$), and odor of implanted female carp vs. 5×10^{-7} M $PGF_{2\alpha}$ (5 times more $PGF_{2\alpha}$ than released by implanted carp). Differences ($P<0.01$) in attraction between a pair of treatments are denoted by **

In our field test, an average of 0.54±0.2 males was found each morning within 20 m of the trap containing the $PGF_{2\alpha}$-implanted carp, and 0.0±0.0 within 20 m of the control trap (Fig. 5a; $P<0.05$). Eight individual males were found near the traps during the course of this experiment, and two of these were found twice. All eight males came from the southeast area of the lake where the traps were located. In contrast, an average of only 0.11±0.1 females was found within 20 m of the pheromone trap, and 0.0±0.0 within 20 m of the control trap (Fig. 5b; $P>0.05$). No carp entered the traps, and no mortality was observed. Analysis of the control data suggested there was no effect of trap location ($P>0.10$).

Discussion

This study showed that $PGF_{2\alpha}$-filled osmotic implants placed into common carp stimulate the release of a sex pheromone complex that is attractive to sexually active conspecific males in both the laboratory and field. To our knowledge, this is the first demonstration of the activity of a PGF-based pheromone in the field. The seemingly normal activity of odor of $PGF_{2\alpha}$-implanted carp, and the fact that it is more active than the $PGF_{2\alpha}$ it contained, demonstrates, as previously hypothesized (Lim and Sorensen, 2011), that the natural pheromone of this species is a synergistic mixture of compounds. The technique of implanting pheromones, and/or their precursors, into fish should be useful in future studies of fish reproductive physiology and pheromone release, as well as in the control of invasive fish species, many of which use known hormonal pheromones whose precursors are commercially available (Sorensen and Stacey, 2004).

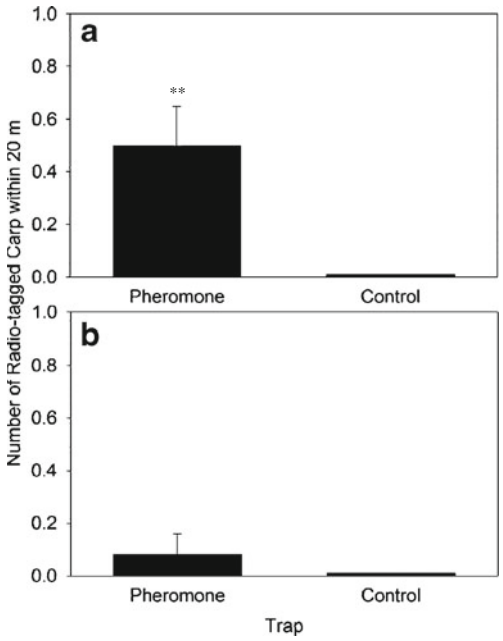

Fig. 5 Daily mean number (±SEM) of: a) radio-tagged male, and b) radio-tagged female common carp within 20 m of pheromone-baited and control traps. Because fewer females (8 vs. 12) than males were radio-tagged, their values were expected to be 30% lower. A difference ($P<0.01$) in mean numbers of fish between a pair of treatments is denoted by **

This study is an important confirmation of our hypothesis that PGF pheromones function as pheromone complexes that include body metabolites. Previous studies have shown that polar body metabolites are discerned and used by carp and goldfish, and may convey species information (Sisler and Sorensen, 2008; Levesque et al., 2011), as well as demonstrating that chemical fractions containing these metabolites synergize the activity of ovulated fish odor (Lim and Sorensen, 2011). However, this study demonstrated that $PGF_{2\alpha}$ only has full activity if added to water within the context of whole body odor. Not only was $PGF_{2\alpha}$-implanted carp odor much more active than $PGF_{2\alpha}$ in both head-to-head tests and dose-response relationships, but it was as attractive as ovulated female odor. Even five-fold increases in $PGF_{2\alpha}$ concentration could not make up for the absence of whole body odor. The PGF metabolite, 15K-$PGF_{2\alpha}$, also appeared to be relatively unimportant (it was not released in large amounts by fully active implanted fish), even though it is detected by the carp olfactory system (Irvine and Sorensen, 1993) and has pheromonal activity in the goldfish (Sorensen et al., 1988, 1989). Similarly, Lim and Sorensen (2011) found that both dh15K-$PGF_{2\alpha}$ and 15K-$PGF_{2\alpha}$ are unattractive to male carp; perhaps these cues are ignored by carp because they strongly characterize female goldfish which, in contrast to carp (Lim and Sorensen, 2011), release them in much greater quantities than they do $PGF_{2\alpha}$ (Sorensen et al., 1995, unpublished results). Interestingly, the amount of $PGF_{2\alpha}$ tested seemed to be unimportant in our assay. However, this may make sense because PGFs are likely released as highly concentrated urinary plumes (Appelt and Sorensen, 2007), whose concentrations would change rapidly and, perhaps unpredictably, in the immediate vicinity of female fish and their body odor.

While our study seems to confirm that carp employ a F prostaglandin-based pheromone complex to mediate spawning attraction, and that body metabolites play a key role in this complex, it is premature to conclude that we have characterized all features and components of the complex. In particular, we have yet to test whether $PGF_{2\alpha}$-implanted fish odor induces full sexual arousal (chasing and nudging) and reproductive priming (elevated levels of luteinizing hormone), as the odor of naturally ovulated goldfish does (Stacey and Sorensen, 2009). The identity of the body metabolite(s) that synergize the action(s) of $PGF_{2\alpha}$ also are unknown, although Levesque et al. (2011) provided evidence that they are polar and likely non-hormonal, because all life stages seem to release them.

Although our proof-of-concept field test was only of 12 days duration, it nevertheless confirmed laboratory studies and demonstrated for the first time that a PGF-based pheromone complex functions in the field. It was especially relevant that males, but not females, were attracted to this sex pheromone, because the pheromone attracts only aroused males (Lim and Sorensen, 2011, unpublished results). This observation also suggests that blank-implanted fish odors would have been ineffective in the field, as they were in the laboratory. While the numbers of fish we found near traps may seem low, each radio-tagged male represented about 70 individuals, so, presumably, the numbers of actual fish attracted were much greater. The 20 m range of attraction fits well with our estimates of the size of the plume that these fish release, and which goldfish are known to release in the laboratory (Stacey and Sorensen, 2009); i.e., the active region is relatively small. Adult carp are notoriously loath to enter traps of any kind. So, while our failure to lure carps into traps was disappointing, it was not unexpected. If pheromonal attractants are used, new, more effective types of traps and/or carp capture devices will be needed. We are now planning to conduct studies that include more controls, higher doses of $PGF_{2\alpha}$ in more fish, and different types of plume structures. The present study is one of just a few to demonstrate the actions of a fish sex pheromone in the field (Johnson and Li, 2010).

This study also sheds new light on the metabolism and clearance of $PGF_{2\alpha}$ in fish. It demonstrates that most $PGF_{2\alpha}$ introduced into carp is cleared directly, with lesser amounts being metabolized to 15K-$PGF_{2\alpha}$ and dh15K-$PGF_{2\alpha}$; there is no evidence of any other biologically active metabolites being produced. This mirrors our earlier results (Lim and Sorensen, 2011) in naturally ovulated carp. In contrast, goldfish injected with $PGF_{2\alpha}$ produce large quantities of 15K-$PGF_{2\alpha}$, which has pheromonal function and dominates the PGF release mixture (Sorensen et al., 1988, 1995). Injection studies in carp should be conducted, as introduction of a bolus might result in different metabolism, although this seems unlikely. Interestingly, our study did not produce any indication that sex of carp influences the production of the PGF pheromone complex. This is of practical significance, because integrated pest management control programs of invasive species may favor (because escape is a concern) use of males in situations in which females are being targeted for removal (Inland Fisheries Service, 2009). The relatively low toxicity of $PGF_{2\alpha}$ to fish is interesting and fortuitous, as mammals appear much more sensitive (Kennedy and Lukash, 1982; Zelinski-Wooten and Stouffer, 1990); perhaps differences in metabolism released to pheromone signaling are responsible.

The implant technique has potential to be developed to study pheromone function in the field, and as a source of environmentally friendly, species-specific fish attractants to either sample rare fishes and/or remove nuisance fishes. Notably, many species of fish, including those of the Family Cyprinidae that includes the highly invasive Asian carps (*Hypophthalmichthys* spp.), appear to use PGF-based pheromones (Stacey and Sorensen, 2009; Lim and Sorensen, unpublished results) and are amenable to this technique. The implant technique not only appears to stimulate fish to produce the natural pheromone, but it functions for extended periods of time, while avoiding complications associated with introducing artificial compounds to waterways. Further studies should explore enhancement, including use of higher doses and the possibility that implanted females may also exhibit sexual behaviors that might make them useful as "Judas fish" decoys (Stacey, 1976; Stacey and Sorensen, 2009; Bajer et al., 2011).

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A Sensitive Analytical Method for Quantifying Petromyzonol Sulfate in Water as a Potential Tool for Population Monitoring of the Southern Pouched Lamprey, *Geotria Australis*, in New Zealand Streams

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Abstract The migratory southern pouched lamprey, *Geotria australis*, is a culturally important fish native to New Zealand. Anecdotal evidence suggests that populations of *G. australis* have declined from historic levels, and presently, this species is rare in many New Zealand rivers and streams. Migratory sea lamprey (*Petromyzon marinus)* use a pheromone mixture to locate suitable spawning sites. This mixture is comprised of three steroids: petromyzonol sulfate (PS), petromyzonamine disulfate (PADS), and petromyzosterol disulfate (PSDS). We examined the migratory pheromone mixture released by *G. australis* ammocetes and found that they excrete predominantly PS. PADS has been detected on some occasions in low concentrations, and PSDS either is not released, or is released in extremely low concentrations. By using a recently developed sensitive mass spectrometry method, we compared passive sampling techniques against more traditional active water sampling as methods for estimating lamprey populations in local streams. Passive sampling provided quantitative data for PS from all sites surveyed, with uptake rates of 0.3 to 45.7 pg/day observed. Conversely, active sampling returned only one positive result out of 19 samples, and with a method detection limit of 2.5×10^{-14} M, this suggests that concentrations of PS in these streams are either extremely low or variable. The combination of passive sampling and triple quadrupole mass spectrometry is a promising tool for monitoring of *G. australis* in New Zealand streams.

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Keywords Migratory pheromone · Petromyzonol sulfate · Petromyzonamine disulfate · Petromyzosterol disulfate · Lamprey · Passive sampling · POCIS · Mass spectrometry

Introduction

The use of pheromones in homing and migration was first suggested by Nordeng (1971, 1977) as a mechanism for controlling natal stream selection in anadromous salmonids. Recent studies with migratory sea lamprey (*Petromyzon marinus*) that do not return to natal streams have shown that migratory pheromones are used as conspecific cues to select suitable spawning streams (Li et al., 2002; Sorensen et al., 2005).

Studies with the sea lamprey suggest that pheromones will be a mixture of compounds rather than just a single molecule or compound (Sorensen et al., 2005; Johnson et al., 2009; Johnson and Li, 2010). The migratory pheromone utilized by adult sea lamprey has been established as a mixture of petromyzonol sulfate (PS), petromyzonamine disulfate (PADS), and petromyzosterol disulfate (PSDS) (Li et al., 1995; Polkinghorne et al., 2001; Fine and Sorensen, 2005; Sorensen et al., 2005; Hoye et al., 2007). Other northern hemisphere lamprey species in the family Petromyzontidae also release PS, PADS, and PSDS (Fine et al., 2004; Baker et al., 2009), and thus this migratory pheromone may not be species-specific.

The southern pouched lamprey, *Geotria australis*, is a culturally important fish species native to New Zealand. Historically it was an abundant and important food source for the indigenous Māori populations (McDowall, 2011). Anecdotal evidence suggests that New Zealand populations of *G. australis* have declined from historic levels (James,

2008). However measuring current population densities is a difficult and laborious task. This is compounded by several factors: many suitable habitats for lamprey are in remote streams; electric fishing techniques can be ineffective at sampling juvenile ammocetes and macrothalmia; and adults are cryptic and seldom seen during their freshwater spawning migration (Jellyman et al., 2002). As such, there is growing interest in efficient techniques for estimating populations in streams as well as for assessing the feasibility of using pheromones to attract adults back to suitable stream habitats for population enhancement.

Previous research has shown that *G. australis* releases PS in high quantities. However, PADS and PSDS were unable to be detected (Baker et al., 2009). Fine et al. (2004) showed that PS was produced and released by 10 larval petromyzontid lamprey species and likely used as part of a common evolutionarily conserved pheromone. With the exception of the Australian lungfish, *Neoceratodus forsteri*, and the African lungfish, *Protopterus aethiopicus* (Hoshita, 1985) - neither of which are present in New Zealand - PS is restricted to lamprey species (Polkinghorne, 1997). Based on observations (i) of high release rates of PS by pouched lamprey ammocetes, (ii) that PS is largely restricted to lamprey species, and (iii) that *G. australis* is the sole lamprey species in New Zealand, this compound may provide a mechanism for monitoring pouched lamprey populations within New Zealand streams. To be effective, pheromones must be released by stream resident fish in concentrations high enough to allow detection by migratory fish at the mouth of streams. However, due to the highly sensitive sensory organs in fish, concentrations can be extremely low (around 10^{-12} M). Detection of such low water concentrations require sensitive analytical techniques that are capable of quantifying pheromone concentrations in natural waters. Liquid chromatography coupled with triple quadrupole mass spectrometry (LC/MS/MS) is suitable for detecting and quantifying environmental concentrations of organic chemicals, and previous work has shown this method - when used in conjunction with pre-concentration - is capable of detecting PS at stream water concentrations as low as 2.5×10^{-14} M (Stewart et al., 2011).

Analysis of fish pheromones from natural water samples (active sampling) usually requires that the chemicals are concentrated, due to the very low (ca. 10^{-12} M or lower) concentration of these compounds within the water. If the chemical is labile in stream water - as is the case with PS (Polkinghorne et al., 2001) - it is necessary to either carry out the concentration step on site to stabilize the chemical or to preserve the chemical on site for later laboratory processing. Both of these approaches require either the transport of toxic chemicals - such as methanol for elution off solid phase extraction (SPE) cartridges, or chemical preservatives. Actively sampling water bodies also may require the transport of large volumes of water, and is likely to be affected by spatial and temporal variations in the concentrations of target chemicals.

Passive sampling is a technique that accumulates chemicals from a matrix (e.g., water or air) over time. It has been used extensively for monitoring environmental micropollutants, especially for less hydrophilic compounds (Stuer-Lauridsen, 2005). However, recent design developments - specifically Polar Organic Chemical Integrative Samplers (POCIS) (Alvarez et al., 2004) - have allowed the concentration of more hydrophilic chemicals from natural waters. Known lamprey pheromones have steroidal backbones, but are all characterized by one or two highly polar ionic functional groups, thus ensuring that they are hydrophilic in nature. As their name suggests, POCIS accumulate chemicals over time and are especially useful where occurrence is transient or concentrations are extremely low (Alvarez et al., 2005), which is the case for chemicals excreted by fish.

Our objectives were to: (1) describe new, highly sensitive analytical techniques to reassess whether or not the migratory pheromone mixture of sea lamprey is also released by *G. australis*; and (2) determine whether PS can be reliably detected at environmental concentrations in streams where populations of ammocetes are known to occur.

Methods and Materials

General Methods

Chemicals All solvents used were of HPLC grade or higher. Synthetic PS was purchased from Toronto Research Chemicals (P293526), while PADS and PSDS were supplied by Professor Peter Sorensen, from the University of Minnesota.

Sorbents C_{18} SPE cartridges were purchased from Phenomenex. Empore discs and Triphasic POCIS samplers were supplied by Dr Etienne Vermeirssen, Eawag, Switzerland. Oasis HLB sorbent (60 μm) and StrataX polymeric sorbent (33 μm) were purchased from Waters Corporation and Phenomenex, respectively, in cartridge format, and sorbent removed from the cartridge prior to preparation of samplers. Polyethersulfone (PES) membranes (Supor®-100; 0.1 μm, 47 mm) were purchased from Pall Corporation.

Passive Sampler Configurations Passive samplers were prepared using aluminium housings, containing a 40 mm diam opening. Sorbents were sandwiched between two PES membranes (47 mm) and secured by stainless steel nuts and bolts. For field studies, protection from debris was achieved by use of a stainless steel mesh (3 mm holes) as an outer casing.

Extraction of Passive Sampler Sorbents and Discs Samplers that had been situated in streams were washed gently with water to remove external debris. Sampler housings were removed, and membranes were pulled apart carefully with stainless steel tweezers. Membranes were placed in a glass funnel, directly over a sintered glass funnel attached to a round bottom flask (100 ml). The membranes were washed with methanol (15 ml), and a vacuum was applied to elute chemicals. The sorbent was washed 4 times with methanol (4×10 ml), and a vacuum was applied after each washing. Methanol was removed by rotary evaporation, and residual water removed by high vacuum. The sample was transferred to a 2 ml amber glass vial in 2×750 μl of methanol. All methanol was removed under a stream of N_2 gas. The sample was re-dissolved in 1:1 methanol:water (100 μl), sonicated briefly, and transferred to a low volume insert inside the 2 ml amber glass vial, and sealed with a lid and septum. Empore discs were removed from their housings and transferred to a scintillation vial. Acetone (7 ml) was added and the disc sonicated for 5 min. The acetone was decanted into a fresh scintillation vial, and the process was repeated with methanol (7 ml). Samples then were processed as described above for passive sampler sorbents.

Concentration of Stream Water for Active Sampling C_{18} SPE cartridges (2 g) were activated with methanol (20 ml) and water (20 ml), prior to sampling. Water samples (400 ml) were eluted through SPE, using positive pressure, created by a syringe, and all eluent was discarded. Cartridges then were removed of excess water by several passes of air, using positive pressure. Material retained by the SPE cartridge was eluted immediately into a glass scintillation vial with methanol (20 ml). The vial was sealed and transferred back to the laboratory. Samples were transferred to round bottom flasks (100 ml) and processed as for passive sampler sorbents.

Mass Spectrometry All quantitation of PS from laboratory passive sampling experiments was performed by liquid chromatography/ion trap mass spectrometry (LC/ITMS) using a Thermo Surveyor system coupled to LCQ Advantage Ion Trap MS. Injections (20 μl) of each were made using a carrier solvent mixture of 1:1 20 mM triethylamine:acetonitrile at a flow rate of 100 μl/min, through a zero volume adaptor, directly into the MS. Detection was by Selected Ion Monitoring (SIM) with the pseudo molecular ion of *m/z* 473.5 ($[M\text{-}Na]^-$) analyzed. Spray voltage used was 5 kV, and a nitrogen sheath gas of 60 psi. A 6 point calibration curve of a PS standard from 0.625 to 10 ng per injection was run with all samples. Manual integration of peak areas was undertaken and PS concentration calculated from a standard curve. Where higher sensitivity was required (i.e., quantitation of PS from passive samplers and active sampling in the field and quantitation of PS, PADS, and PSDS from lamprey ammocete tankwater), LC/MS/MS was used, as described previously (Stewart et al., 2011). This method afforded instrument practical quantitation limits of 0.05, 0.05, and 0.50 ng/ml for PS, PADS, and PSDS, respectively.

Experiment 1 How Does the Chemical Signature for *Geotria australis* Differ from the Northern Sea Lamprey, *Petromyzon marinus*?

Lamprey Ammocete Tank Holding Conditions Southern pouched lamprey were held in two 35 l tanks in 18°C aged filtered tap water (30 ammocetes/tank). Tanks contained 15 l of water with 10 cm of sand at the base. Filter pumps were run on each tank to maintain water quality and create a slow current flow. Ammocetes were given 1 wk to acclimate to their new tank environment before experiments began. Ammocetes were fed once per week on yeast. For feeding, filter pumps were turned off, and 30 g baker's yeast were added to the water. After 24 h, a complete water change was conducted to remove old water and uneaten yeast, and the tanks were refilled with aged filtered tap water. Tanks then were left with no filtration for between 12 and 16 h, to allow pheromone accumulation before collecting water samples for analysis. Each tank was sampled on 6 separate occasions, from 23rd September to 10th December 2010.

Quantitation of PS, PADS and PSDS from Ammocete Tank Water (LC/MS/MS) At a defined time after a water change (ca. 15 h), 400 ml of tank water were removed from each holding tank. A C_{18} SPE cartridge (2 g, Phenomenex) was conditioned with methanol (20 ml) and H_2O (20 ml) and water eluted through SPE, using positive pressure at a flow that is fast enough to form rapid dripping of the eluent at the end of the cartridge, but not a constant stream. The water sample was eluted fully through the SPE cartridge, and all eluent was discarded. The cartridge then was removed of excess H_2O by several passes of air through with positive pressure. The material retained by the SPE cartridge was eluted immediately into a glass scintillation vial with methanol (20 ml). Final work-up of the sample was as described previously for passive sampler sorbents and discs. The sample was sealed with a lid and septum for LC/MS/MS analysis as described previously (Stewart et al., 2011).

Experiment 2 What is the Uptake of PS by Different Samplers in Pure Water? Polypropylene ice cream containers (10×2 l) and lids, were washed and dried. Nanopure water (1 l) was added to each of the 10 containers, followed by 50 μg of PS (in 50 μl of methanol). Lids were attached, and the container contents were gently mixed on a shaker table for 30 min. Sampler configurations were Oasis (Pharmaceutical) POCIS, Triphasic (Pesticide) POCIS, Empore C18,

and Empore SDB-RPS, with each carried out in duplicate. Empore discs were pre-soaked in methanol (30 min) followed by water (30 min, then 15 min) to activate them. Each sampler was placed in a separate container, and immediately an aliquot of water (1 ml) was removed as a time 0 measurement. The shaker table was set at 25 rpm, and aliquots were removed for analysis at time intervals of 2, 4, 6, 24, 70, 96, and 144 h. The sorbents were extracted and analyzed as described under general methods.

Experiment 3 What is the Uptake of PS by Different Samplers in Raw Water? In addition to the 3 most efficient samplers from experiment 2 (Empore C18, Empore SDB-RPS, and POCIS Oasis), a fourth sampler configuration, using Strata-X sorbent, was trialed. The four passive sampler configurations were placed in a circular tank (40 cm diam), containing 7.5 l of raw water (filtered and aerated tap water) with a PS concentration of 10^{-8} M. The tank was placed in a controlled temperature room (15°C). Empore discs were pre-soaked in methanol (30 min) and water (30 min) and sandwiched between 2 PES membranes. Oasis and Strata-X passive samplers were made by encasing 200 mg of sorbent between two PES membranes and clamping in stainless steel samplers. A submersible pump was placed within the tank to provide a current flow to simulate stream conditions. Water was changed daily, with PS added after each water change (37.2 μl of a stock solution of 1 mg/ml in methanol) to achieve a concentration of 10^{-8} M. The samplers were left *in situ* for 5 d, before removing and analyzing the concentration of PS on each sorbent. The sorbents were extracted and analyzed as described under general methods.

Experiment 4 Can PS be Protected from Microbial Breakdown by Sampler Membranes? PS was pre-adsorbed onto Oasis and Strata-X sorbents by adding 25 μg of PS stock solution (1 mg/ml in methanol) per 200 mg of sorbent, followed by enough methanol to completely wet the sorbent. This was mixed and left in a heating block at 25°C in a fume cupboard overnight. A subsample of each sorbent (200 mg) was weighed into a glass scintillation vial, nanopure water (250 μl) added, and the phase mixed to a thick paste. Two replicates of Oasis and Strata-X samplers were made, while control samples of sorbent in scintillation vials were placed in the controlled temperature room (15°C) where the experiment was carried out. The tank configuration was identical to experiment 3. Raw water (7.5 l) was added, and the samplers left for 5 d. The sorbents were extracted and analyzed as described under general methods.

Study Sites Three streams (Kaniwhaniwha Stream, Styx River, and Pigeon Bay Stream) were chosen for active water sampling and passive sampler deployment based on densities of ammocetes recorded by the New Zealand Freshwater Fish Database. The Kaniwhaniwha Stream is located in the North Island of New Zealand (mid stream site E2694123:N6365477) and represents a large stream system where recent database records suggest ammocete densities are low. Styx River (mid stream site E2482790:N5749735) and Pigeon Bay Stream (mid stream site, E2501900: N5722515) are small stream systems located in the South Island of New Zealand with high abundances of ammocete in recent database records.

Experiment 5 Comparison of Passive Sampling with Active Sampling

Passive Sampler Deployment Passive samplers containing Oasis sorbent (200 mg) were protected from stream debris by stainless steel mesh and attached to a Waratah (metal stake) that had been driven into the stream bed. The Waratah was in turn fastened to the stream bank for security. Samplers were situated in the main flow of the stream at mid water depth under base flow conditions. Samplers were left *in situ* for 29 d (Kaniwhaniwha Stream) or 21 d (Styx River and Pigeon Bay Stream) before removal. Upon collection, all samplers were placed in a separate plastic bag and stored on ice prior to processing. Samplers were deployed within Kaniwhaniwha Stream from 21st January to 19th February 2009. In total, 6 samplers were deployed at three locations (Table 1). Trials within the Styx River and Pigeon Bay Stream were undertaken between 2nd and 23rd of February 2010. Four sites were sampled within the Styx River, with 1 sampler deployed at each site. One sampler also was deployed at each of five sites within Pigeon Bay Stream.

Active Sampling Water was sampled from all three sites of Kaniwhaniwha Stream where passive samplers were deployed, with two replicates taken per site. Each site was sampled on two occasions, on 21st and 24th September 2010. Pigeon Bay Stream and Styx River were sampled once on 24th November 2010. The site within each stream that had the highest concentration of PS using passive samplers was chosen for active sampling. Five samples were taken along a transect across site 4 of Styx River, three samples taken along a transect across site 3, and five samples taken along a transect across site 4 of Pigeon Bay Stream.

Experiment 6 What is the Temporal and Spatial Variability of PS Uptake from Passive Samplers across a Stream? Three passive samplers were placed across Kaniwhaniwha Stream at the survey site where previous sampling had found the highest concentrations of PS (site 1). The true right sampler was placed on the original Waratah in the main flow of the stream, a second sampler was placed in the

Table 1 Comparison of passive sampling (petromysonol sulfate uptake) and active sampling (petromyzonol sulfate water concentration) from the 3 streams studied

Site	Site description	Coordinates		Passive sampling	Active sampling
		Easting	Northing	PS uptake (pg/day)	PS Concentration (x 10^{-14} M)
Styx River 1	River mouth	2484995	5756370	1.3	NT
Styx River 2	Downstream	2482790	5749735	3.2	NT
Styx River 3	Upstream lower	2479279	5749112	4.1	NT
Styx River 4	Upstream upper	2479115	5748845	45.7	<2.5[a]
Pigeon Bay 1	River mouth	2501555	5724300	0.7	NT
Pigeon Bay 2	Lower stream	2601715	5723364	0.3	NT
Pigeon Bay 3	Hayes reserve	2501900	5722515	2.2	<2.5–7.4[b]
Pigeon Bay 4	Innes Road	2501900	5721820	9.1	<2.5[a]
Pigeon Bay 5	Pigeon Bay Road	2501910	5721050	0.3	NT
Kaniwhaniwha 1	Midstream	2694123	6365477	15.7	<2.5[c]
Kaniwhaniwha 2	Upstream	2693153	6362642	2.4	<2.5[c]
Kaniwhaniwha 3	Downstream	2698488	6368027	3.0	<2.5[c]

[a]range of 5 transects across the stream

[b]range of 3 transects across the stream

[c]average of 2 replicates

NT=not tested

middle of the stream, and a third sampler placed near the true left stream margin. The three samplers were left *in situ* for 7 d before collection. In total, four replicates (1 per wk) were undertaken on consecutive weeks from 2nd February to 2nd March 2011. An Onset® tidbit temperature logger and Odyssey® capacitance water level recorder were deployed at the sampling site prior to initiating the passive sampler trial. To determine stream flow, a stream gauging was undertaken on deployment of the recorder and in each week of the trial when samplers were collected and replaced. Upon collection, samplers were placed on ice prior to the return to the laboratory, and extracted as previously described.

Data Analysis Kruskal-Wallis ANOVAs and Mann–Whitney *U* tests were utilized to identify significant differences between PS uptake on sorbents.

Results

Experiment 1 Does the Chemical Signature for *Geotria australis* Differ from the Northern Sea Lamprey, *Petromyzon marinus?* An average release rate of 0.30±0.10 ng/larvae/h was established for PS, and 0.0010±0.0004 ng/larvae/h for PADS. PSDS was not detected in any sample.

Experiment 2 What is the Uptake of PS by Different Samplers in Pure Water? The two Empore discs had the most rapid uptake of PS (Fig. 1). Empore C18 had effectively removed all detectable amounts of PS from the water after 70 h, while for Empore SDB-RPS this was achieved at 144 h. Oasis was the best of the two POCIS configurations, with PS concentration reduced to 29% of the original concentration after 144 h. The triphasic POCIS configuration was the least efficient, with 43% of the original PS still present in the water after 144 h. The control data show some initial loss of PS, but this leveled off to around 85% of the initial concentration during the experiment.

PS was quantified in each disc or sorbent (Fig. 2a). These results revealed that Triphasic was the least efficient in removing PS from pure water, whereas the uptake of PS by Oasis and the Empore discs were not significantly different. The larger error bars for Oasis were due to partial spilling of sorbent from one replicate when removing the membranes, and therefore a lower amount was extracted from that sample.

Experiment 3 What is the Uptake of PS by Different Samplers in Raw Water? In raw water, where microbial degradation of PS is present, the Oasis configuration was the most efficient at concentrating PS, with effectively twice the amount concentrated than the other samplers (Fig. 2b).

Experiment 4 Can PS be Protected from Microbial Breakdown by Sampler Membranes? In the PS protection trial, a virtually identical performance was found between Oasis and StrataX sorbents with 0.67 and 0.66 μg/cm^2 retained, respectively. Controls were 0.93 and 0.71 μg/cm^2, for Oasis and StrataX sorbents, respectively (Fig. 2c). There were no significant differences between control and experimental trials with respect to the amount of PS retained by sorbents.

Experiment 5 Comparison of Passive Sampling with Active Sampling. Uptake rates of PS in the field by passive samplers are presented in Table 1. All passive samplers recorded

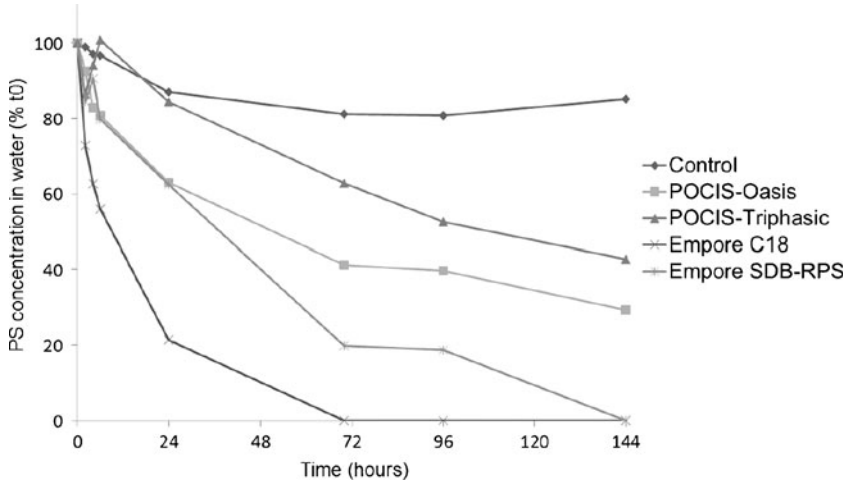

Fig. 1 Comparison of four passive sampling phases for concentration of petromyzonol sulfate from nanopure water

some uptake of PS, with a range of 0.3 to 45.7 pg/day. Active sampling at 6 of these sites detected PS in only one sample; with 7.4 x 10^{-14} M recorded from one transect at Pigeon Bay, site 3 (Table 1).

Experiment 6 What is the Temporal and Spatial Variability of PS Uptake from Passive Samplers across a Stream? A high level of spatial and temporal variability in PS uptake rates was observed among the three samplers. The samplers afforded average PS uptakes of 57±28, 22±4, and 16±4 pg/day, for true right, middle, and true left samplers, respectively (Fig. 3). There was no significant difference in PS uptake among sampler locations. Although the true right sampler was placed within the main stream flow, this sampler did not absorb significantly higher levels of PS compared to the samplers placed in the middle and true left side of the stream. The high variability in uptake rates of PS among sampling occasions was not related to changes in stream flow, as flow was relatively stable throughout the survey period, with maximum daily flows ranging between 0.5 and 2 m^3 s^{-1} during the sampling period. The average daily water temperature also was similar over the 4 week survey ranging between 17.3 and 20.0°C.

Discussion

Petromyzonol sulfate was the most abundant pheromone released by ammocetes of *G. australis*, with PADS released in very low concentrations (0.33% of PS), and PSDS either was released in extremely low concentrations, or not at all. The release rates reported here for PS of 0.30±0.1 ng/larva/h are markedly lower than those reported previously from *G. australis* of 59.6±10.9 ng/larva/h (Baker et al., 2009). The lower release rate in this present study may be a result of reduced feeding by ammocetes, which in turn would reduce the release of the bile acid. Reduced feeding could be in part due to the shorter acclimation time for ammocetes in this experiment (1 wk) compared to 3 months previously (Baker et al., 2009). Our data were acquired on a triple quadrupole mass spectrometer (MS/MS), whereas previous data were acquired on an ion trap mass spectrometer (ITMS). The MS/MS has orders of magnitude sensitivity improvement over the ITMS, which is highlighted by the detection of PADS, even though release rates were much reduced. Furthermore, MS/MS is able to fragment PS, whereas ITMS is not, thus providing selectivity and improved confidence in chemical confirmation (Stewart et al., 2011). As such, MS/MS appears to be more applicable to quantifying pheromones at the extremely low water concentrations observed in the environment.

Within the family Petromyzontidae, *G. australis* is the sole member of the subfamily Geotrinnae, and occurs only in the southern hemisphere. As such, evolution of the migratory pheromone for *G. australis* could be expected to be different from that found for Northern Hemisphere lamprey species. The three Northern Hemisphere lamprey species studied to date; the American brook lamprey (*Lethenteron appendix*), Northern brook lamprey (*Ichthyomyzon fossor*), and the sea lamprey, have all been found to release PS, PADS, and PSDS (Baker et al., 2009). That PADS and PSDS are released by *G. australis* ammocetes in extremely low proportions, or not at all, suggests that these chemicals are not important migratory pheromones for this species. Extensive behavioral and physiological studies of the migratory pheromone for *G. australis* are yet to be carried out. Therefore, it cannot be ascertained at present whether PS is the major component of the migratory pheromone for this species. Nevertheless, PS is consistently released by *G. australis* ammocetes, is almost exclusively restricted to lamprey species (with the two exceptions not found in New Zealand), so could be a useful tool for population detection and monitoring.

Within the laboratory, Empore discs were the most efficient at concentrating PS from water, presumably because

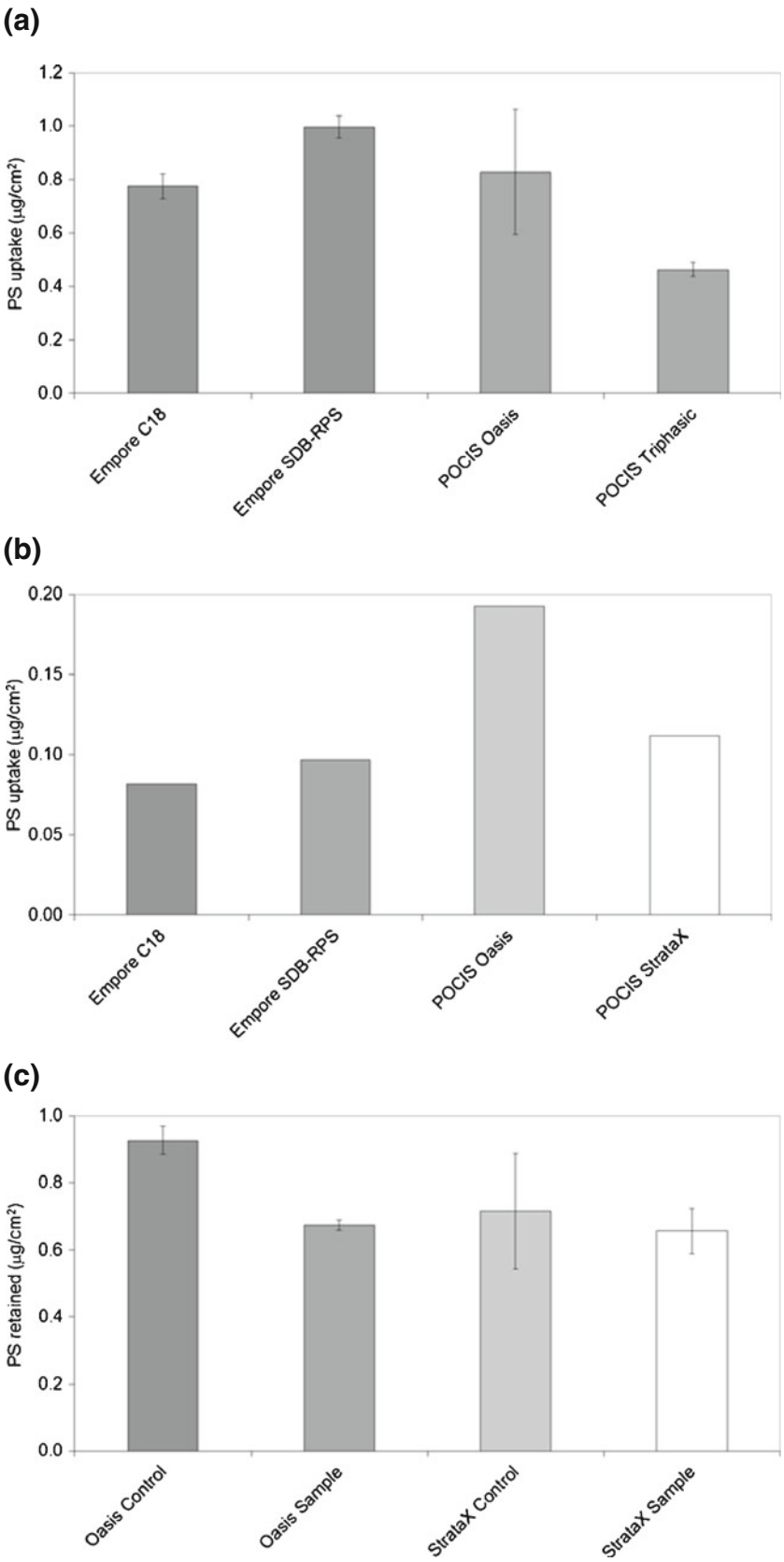

Fig. 2 Petromyzonol sulfate uptake by each sorbent (µg/cm^2) after 6 d in nanopure water (**a**) or 5 d in raw water (**b**). Petromyzonol sulfate retained on samplers in protection trial (**c**). Error bars represent ±1 standard error from 2 replicates

they were not encased in PES membranes, which would slow the transfer rate of a chemical onto the sorbent. However over 6 days, the amount of PS taken up by the Oasis sorbent was similar to the Empore discs. When all

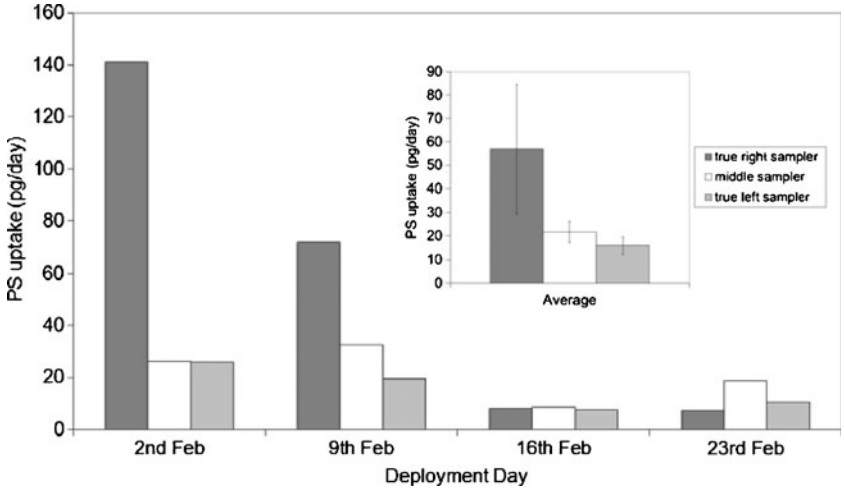

Fig. 3 Temporal variability in petromyzonol sulfate across three transects at site 1 of Kaniwhaniwha Stream. Inset are average data with error bars representing ±1 standard error

samplers were encased in a PES membrane, Oasis outperformed the other samplers. These data show that when the PES membrane is taken into account, the two Empore discs are not as efficient at concentrating PS. Furthermore, Empore discs require pre-activation with methanol prior to deployment, so are not ideally suited for use at remote sites, where transport of toxic methanol is necessary.

Earlier studies have shown that PS degrades rapidly in raw water due to microbial breakdown, with a half-life of 1.5 days (Polkinghorne et al., 2001). Any final passive sampling configuration will require that PS is protected from microbial breakdown while the sampler is in the stream. Polyether sulfone (PES) membranes are the most effective for long term use in integrative samplers (Alvarez et al., 2004) and, with a pore size of 0.1 μm, will prevent microbial infiltration of the sorbent and the associated breakdown of PS.

The stability trial revealed no significant difference between Oasis and StrataX sorbents. POCIS are a configuration of passive samplers designed to act as infinite sinks for moderately polar (Log K_{ow}<4) chemicals (Alvarez et al., 2004). These data suggest that, for PS at least, there is little difference between the two phases, and that either will perform well as integrative samplers. However, as Oasis outperformed StrataX in the raw water uptake experiment and is a standard sorbent used in POCIS, it was deemed the more appropriate sorbent for sampling PS within natural waters.

The present study illustrates the advantages of passive over active sampling (Table 1). The sample concentration and LC/MS/MS method will detect PS in stream water down to 2.5×10^{-14} M (Stewart et al., 2011), so the lack of results for active sampling can either mean the concentration of PS in the stream is very low (<2.5×10^{-14} M) and/or concentrations are highly variable.

Electrophysiological studies have shown that adult sea lamprey can detect PS at concentrations of 10^{-12} M, with only moderate behavioral activity at 10^{-11} M (Li et al., 1995; Li and Sorensen, 1997). Therefore, a stream water concentration of less than 10^{-12} M of PS has been considered to be below the detection limit of adult sea lamprey and not biologically relevant as a migration cue (Fine and Sorensen, 2005). Measurements of PS at picomolar concentrations were achieved in streams known to contain larval lampreys and to attract migratory adults, while not measured in streams lacking ammocetes (Fine and Sorensen, 2005). The analytical method used by Fine and Sorensen (2005) had a detection threshold of 10^{-12} M, which is 40 times less sensitive then the detection limit in the present study of 2.5×10^{-14} M. Therefore, given the detection threshold of the current LC/MS/MS analyses, it is surprising that PS was not found in more active water samples, and suggests that within the study streams the concentration of PS is extremely low.

Based on the results of both passive and active sampling (Table 1), the spatial differences across sampling sites suggest that the distribution of PS within a stream is highly variable. This could be explained in part if the water samples were taken close to communities of ammocetes where pheromone plumes are released and thus have not had time to become completely mixed within the flow. This seems a likely scenario at Pigeon Bay Stream, where only one of five samples across a stream ca. 2 m wide, had detectable concentrations of PS. For this reason, passive sampling appears the more appropriate mechanism for determining if stream populations of lamprey ammocetes are present in high or low abundance.

Spatial and temporal variability in the distribution of PS within streams will need to be accounted for in using passive sampling as a population monitoring tool. Future work is planned to survey populations of pouched lamprey ammocetes within streams and determine if results from passive samplers can be correlated to total stream abundance estimates.

The potentially extremely low concentrations (ca. 10^{-14} M or lower) of pheromones in the stream waters tested have important implications for population monitoring and restoration of *G. australis* within New Zealand. If ammocete populations are below a critical threshold in certain streams so that they are not releasing adequate concentrations of migratory pheromones, adults may not locate these suitable spawning streams, and the abundance of lamprey within such waterways could continue to decline. This potentially could be addressed by "seeding" appropriate streams with synthetic pheromone standards, or populations of ammocetes, to attract adults during the spawning season. However, further knowledge of the compounds used by *G. australis* as migratory pheromones is required before this could be achieved.

As a field tool, passive sampling has several advantages over active sampling. Samplers can be pre-prepared in the laboratory prior to their use in the field. They contain non-toxic chemicals, are a stable configuration, and they are small and easily transported to remote sites. The casings can be reused, so they are cost effective, and they do not require specialized training to be deployed. When secured with wire mesh in a stream setting, they are remarkably strong, surviving for weeks, even through high rainfall events (authors' unpublished data). The samplers act as an "infinite sink" for polar and moderately polar chemicals in water matrices, so are ideally suited for water soluble pheromones.

The combination of triple quadrupole mass spectrometry and passive sampling is a powerful and tunable technique for measuring lamprey pheromones in natural stream waters. To our knowledge this is the first time this technique has been developed for any naturally occurring environmental chemical. However, for this methodology to become a useful tool for monitoring *G. australis* ammocete populations, further validation is required. Variability of uptake of PS (and other chemicals of interest) by the sampler needs to be addressed, differential release of PS by lamprey needs to be determined, and uptake data need to be correlated with extensive population surveys in streams with a wide variety of lamprey densities.

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Investigation of Scents on Cheeks and Foreheads of Large Felines in Connection to the Facial Marking Behavior

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Abstract We investigated head- and cheek-rubbing behavior in four species of large felines, lions (*Leo panther*), leopards (*Panthera pardus*), tigers (*Panthera tigris*), and cougars (*Puma concolor*), in captivity. Preliminary behavioral observations found that lions and tigers, but not leopards and cougars, showed behavioral responses to cardboard rubbing samples from head and cheek areas from conspecific felines, compared to the blank cardboard controls. In this context, surface samples on the facial areas of each species were collected to analyze volatile organic compounds that could be involved in the facial marking of felines. Previously developed stir bar surface sampling methodology was used. From all cheek and forehead samples, 100 volatile organic compounds were identified or tentatively identified. Among these, 41 have been previously reported to be present in feline urine and marking secretions. Several new compounds were identified on facial surfaces. Some of the compounds showed substantial quantitative differences among the species. One compound, that has not been reported previously in mammals, 3-acetamidofuran, was found in all investigated species. It was synthesized and tested for behavioral responses. No responses were elicited in a preliminary test. Future research will test other potential signaling compounds and their mixtures for ability to elicit behavioral responses.

Keywords Felidae · Facial marking behavior · Chemical signals · Lion · Tiger · Cougar · Leopard · Volatile compounds · Gas chromatography–mass spectrometry

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Introduction

Members of the family *Felidae* are essentially solitary in their activities and rarely associate with each other, with the exception of lions, cheetahs, and domestic cats (Kleiman and Eisenberg, 1973). This asocial existence does not eliminate the need for communicative signals. Individuals still need to avoid conflict and find mates, and use a complex repertoire of auditory calls and roars (Schaller, 1972), visual signals, including dirt scrapes and tree scratches (Wemmer and Scow, 1977; Smith et al., 1989), and olfactory marks deposited in the form of urine and feces, to convey information (Schaller, 1972; Kleiman and Eisenberg, 1973; Smith et al., 1989).

Marking territory with urine and feces is thought to be a way in which important volatile compounds (signals) identify individuals and advertise reproductive condition (Schaller, 1972). Urine and anal sac-derived volatile compounds have been partially characterized for many large cats, including tigers (Brachmachary and Dutta, 1981; Brachmachary et al., 1992; Poddar-Sarkar et al., 1994, 2004; Poddar-Sarkar, 1996; Burger et al., 2008), lions (Albone and Eglington, 1974; Anderson and Vulpius, 1999), leopards (Brachmachary and Dutta, 1984; Poddar-Sarkar and Brahmachary, 2004),

cheetahs (Burger et al., 2006), and bobcats (Mattina et al., 1991).

A second marking behavior, not as well understood, is head and cheek rubbing. Rubbing behavior has been documented in several species of captive small cats (Mellen, 1993), domestic cats (Van Den Bos and de Cock, 1994; Penny Bernstein personal communication 2009), wild lions (Schaller, 1972), wild leopards (Bothma and le Riche, 1995), and wild tigers (Smith et al., 1989), but the function of the rubbing is in question. Some suggest the behavior functions as a mechanism to pick up scent from objects or as a visual display (Rieger, 1979). Others suggest that rubbing deposits scent, as well as picking up scent and displaying visual signals (Mellen, 1993). Some volatile compounds, such as long-chain carboxylic acids, in particular, have been identified from lion manes (Poddar-Sarkar et al., 2007). In both domestic cats and lions, there is believed to be a form of tactile communication, as well as a marking behavior, which may serve the same purpose as grooming in primates (Schaller, 1972; Van Den Bos and de Cock, 1994).

The purpose of our study was threefold. We collected facial samples on cardboard squares from captives of four species of large felines, lions (*Leo panther*), leopards (*Panthera pardus*), tigers (*Panthera tigris*), and cougars (*Puma concolor*) to observe whether the samples could elicit behavioral responses from conspecifics, indicating that possible chemical signaling compounds would be transferable. Second, we collected separate samples from the facial areas of felines to determine the volatile organic compounds that are present in the head and cheek areas, and could possibly be involved in the marking behavior. Third, we tested behavioral responses to an unusual compound present on all the feline species.

Methods and Materials

Study Site We conducted this study with captive felines at the Exotic Feline Rescue Center, Center Point, Indiana, USA. This is a 108-acre facility that houses 205 felines, representing 10 species. The cats are housed in fenced enclosures ranging from 100 to 1,600 m^2, depending on the species and numbers of individuals housed together. Each enclosure contains at least one climbing tower, water trough or pond, and shelter boxes with straw. Cats are fed a diet that includes raw meat and provided with Boomer Balls™ (Domestic, Zoo and Exotic Animal Products) and other toys for behavioral enrichment. Male lions are vasectomized to retain manes. Other individuals are spayed or neutered, depending upon enclosure members, to prevent pregnancies, and so both altered and unaltered individuals are included in the population. The study was approved by Bloomington Institutional Animal Care and Use Committee Protocol, Study # 07–039.

Preliminary Scent Collection for Behavior Tests To determine if active compounds could be transferred from the head and cheek areas, samples for behavioral tests were taken by rubbing 10×10 cm generic white paper-based cardboard squares on the head and cheek areas of an unneutered male of each species. Samples were collected in May 2008, stored in plastic bags, and refrigerated. Tests were conducted from 5/15/2008 to 9/10/2008 between the hours of 9 am and 1 pm. Three squares, a blank to control for handling contamination, a head or a cheek sample, were mounted with tape on enclosure fences approximately 1.5 m apart at eye level for each species. Ten minutes of observation began when the first individual approached the squares, and subsequent responses were recorded. Positive responses included sniffing, flehmen, licking, rubbing, and urinating toward the swatch. Twenty seven 10-min sessions were conducted with the animals listed in Table 1. Some enclosures had more than one individual. In those cases, it was possible that one animal could be responsible for most of the responses; however, these observations were carried out as preliminary tests to determine if volatile compounds that were able to elicit responses were present. Responses to head or cheek samples were compared to that to the control square by χ^2 tests of frequencies.

Table 1 Animals used for cardboard square scent tests

Species	Enclosure occupants	Males/Females
Cougar	single	male
Cougar	single	female
Cougar	two	1 male and 1 female
Leopard	single	male
Leopard	two	1 male and 1 female
Leopard	two	female
Leopard	two	1 male and 1 female
Leopard	three	1 male and 2 females
Lion	three	males
Lion	single	female
Lion	single	male
Lion	seven	females
Lion	two	1 male and 1 female
Lion	single	male
Lion	single	female
Lion	single	female
Tiger	four	2 males and 2 females
Tiger	four	2 males and 2 females
Tiger	single	male
Tiger	four	2 males and 2 females
Tiger	four	1 male and 3 females
Tiger	five	2 males and 3 females
Tiger	two	1 male and 1 female

Feline Forehead and Cheek Surface Sampling for the Chemical Analyses After positive preliminary responses were obtained using the cardboard squares, separate samples for chemical analysis were gathered using a polymer-coated, 1 cm long rolling pin (Twister™ stir bar, Gerstel GmbH, Mülheim an der Ruhr, Germany). Information about the felines used for the facial stir bar scent collections is shown in Table 2. This technique was developed previously for *in situ* surface-sampling applications (Soini et al., 2006). Surface samples were collected in July 2008 from the head (above eyes and between ears) and cheek areas (near the dorsal edge of the mouth) of 21 individuals. These included 3 males and 3 females each of lions, tigers, and cougars, and 1 female and 2 male leopards. A preconditioned stir bar with an embedded internal standard was placed between the jaws of the collection device. Two separate 5-cm long stretches of the sampling area were rolled over with a stir bar (10 cm^2 skin area). The stir bar was subsequently dropped from the collection device and placed in a capped Twister™ glass vial. All samples were stored in the refrigerator for 2–3 days until analysis. In previous human skin studies, the collected skin surface samples were chemically stable up to 14 days (Soini et al., 2006; Penn et al., 2007).

Reagents and Materials for Chemical Analyses Standards were purchased from Sigma-Aldrich Chemical Company (Milwaukee, WI, USA) and TCI America (N-acetylglucosamine; Portland, OR, USA). Stir bars (Twister™, 10 mm, 0.5 mm film thickness, 24 µl polydimethylsiloxane) used for sample collection were obtained from Gerstel GmbH (Mülheim an der Ruhr, Germany). The stir bars were conditioned prior to embedding the internal standard in a TC-2 tube conditioner (Gerstel GmbH) at 300°C under high helium stream.

Table 2 Housing, sex and age information for felines used for stir bar facial scent collections for chemical analyses

Subject	Age	Species/Sex	Housing condition
Autumn	16	Cougar/Female	single
Ben	9	Cougar/Male	with 1 female
Tinker	unknown	Cougar/Male	single
Aries	unknown	Cougar/Male	single
Paco	unknown	Leopard/Female	single
King	unknown	Leopard/Male	with 1 female
Rodney	3	Leopard/Male	with 1 female and 1 male
Jackie	5	Tiger/Female	with 2 females and 4 males
Anna	5	Tiger/Female	with 2 females and 1 male
Samara	5	Tiger/Female	with 2 females and 1 male
Tony	unknown	Tiger/Male	single
Max[a]	2	Tiger/Male	with 1 male lion
Casey	8	Tiger/Male	with 3 females
Lauren	5	Lion/Female	with 1 female and 1 male
Gaby	10	Lion/Female	with 3 females and 1 male
Nala	10	Lion/Female	with 3 females and 1 male
Clancy	4	Lion/Male	with 1 female
Petey	8	Lion/Male	single
King	8	Lion/Male	with 2 females

[a] Only one housed with a different species

Standards, 3-acetamidofuran (3-AF) and 3-acetamido-5-acetylfuran (3-A-5AcF), were synthesized by adapting the procedures of C-N cross-coupling methodology, as reported by Padwa et al. (2003). Thus, reactions of 3-bromofuran and 5-acetyl-3-bromofuran (Antonioletti et al., 1985) with acetamide were catalyzed by CuI (10 mol%) in 1,4-dioxane at 110°C. The furan products were isolated following flash silica gel chromatography and were fully characterized by spectroscopic and mass spectral data. Proton and carbon NMR spectroscopy confirmed the assigned structures of 3-AF and 3A-5AcF, by comparison with data from other laboratories (Cambell et al., 1982; Franich and Goodin, 1984). Proton (^{1}H NMR, 400 MHz) and carbon (^{13}C NMR, 101 MHz) spectra were recorded in $CDCl_3$ and used to establish sample purity at >95%. This was further confirmed by subsequent HPLC analysis.

Preparation of 3-Acetamidofuran (3-AF) To a sample of CuI (29 mg, 0.15 mmol, 10 mol%) and K_2CO_3 (890 mg, 6.5 mmol) under argon, was added 1,4–dioxane (10 ml) followed by N,N'-dimethylethylenediamine, 3-bromofuran (234 mg, 1.6 mmol) and acetamide (100 mg, 1.6 mmol). The reaction was heated at 100°C for 24 hr, cooled, and then diluted with dichloromethane (10 ml). After filtration through a plug of silica gel, the mixture was concentrated under reduced pressure, and the crude product was purified by flash silica gel chromatography to give 156 mg (1.25 mmol: 80%) of 3-acetamidofuran, which was confirmed spectroscopically by comparisons to samples prepared by other laboratories (Cambell et al., 1982; Padwa et al., 2003): mp 91–92°C; IR ($CHCl_3$) υ=3,450, 1,675 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.5 (s, CH_3), 6.32 (dd, J=1.5 Hz; J= 0.7 Hz), 7.29 (t, J=1.5 Hz; J=1.5 Hz), 7.65 (br, s; N-H), 7.99 (dd, J=1.5 Hz; J=0.7 Hz), ^{13}C NMR ($CDCl_3$) δ 23.5, 104.8, 124.4, 132.7, 141.5, 168.0; High resolution mass spectrum (HRMS), *m/z* calculated for $C_6H_7O_2N$ (M^+) 125.0471, found 125.0475.

Preparation of 3-Acetamido-5-acetylfuran (3A-5AcF) To a sample of purified CuI (20 mg, 0.10 mmol, 10 mol%) and K_2CO_3 (590 mg, 4.3 mmol) under argon, was added 1,4-dioxane (3 ml) followed by N,N'-dimethylethylenediamine, 5-acetyl-3-bromofuran (190 mg, 1.0 mmol), and acetamide (72 mg, 1.2 mmol). The mixture was heated at 110°C for 24 hr, cooled to 22°C, and diluted with dichloromethane

(10 ml). After filtration through a plug of silica gel, the mixture was evaporated under reduced pressure, and the crude product purified by flash silica gel chromatography to give 98 mg (52%) of 3-acetamido-5-acetylfuran, which was identified and confirmed spectroscopically by comparison with data obtained from a sample previously prepared by another method (Franich and Goodin, 1984): IR ($CHCl_3$) 1690–1670 (broad C=0) cm^{-1}: ^1H NMR ($CDCl_3$) δ 2.13 (s, CH_3), 2,45 (s, CH_3), 7.71 (s,H), 8.15 (s, H), 8.32 (br s, N-H); ^{13}C NMR ($CDCl_3$) δ 23.2, 25.8, 110.2, 126.7, 136.2, 150.6, 168.3, 187.5; HRMS *m/z* calculated for $C_8H_9O_3N$ (M^+) 167.0577, found 167.0574.

Preparation of the Embedded Internal Standard An internal standard, 8 ng of 7-tridecanone (Aldrich, Milwaukee, WI, USA) was added in 5 μl of methanol to pre-cleaned 20 ml vials containing 2.0 ml of high-purity water (OmniSolv® EM Science, Gibbstown, NJ, USA), followed by addition of a preconditioned stir bar. Stirring speed was 850+ rpm on a Variomag Multipoint HP 15 stirplate (H + P Labortechnic, Oberschleissheim, Germany). Prior to extraction, all glassware was washed with acetone and dried at 80°C. After extraction, stir bars were stored for up to 3 days in individual vials inside a refrigerator prior to sample collection. The embedded internal standard is stable in the refrigerator for up to 20 days (Soini et al., 2006).

Analytical Instruments The gas chromatograph-mass spectrometer (GC-MS) used for analysis was an Agilent 6890N gas chromatograph connected to a 5973i MSD mass spectrometer (Agilent Technologies, Inc., Wilmington, DE, USA), with a Thermal Desorption Autosampler and Cooled Injection System (TDSA–CIS 4 from Gerstel). Positive electron ionization (EI) mode at 70 eV was used with a scanning rate of 2.41 scans.sec^{-1} over the range of 40–350 amu. The MSD transfer line temperature was set at 280°C, and the ion source and quadrupole temperatures 230°C and 150°C, respectively. A DB-5MS (30 m×0.25 mm, i.d., 0.25 μm film thickness) capillary column from Agilent (J&W Scientific, Folsom, CA, USA) was used for separations. Samples were thermally desorbed in a TDSA automated system, followed by injection onto the column with a cooled injection assembly, CIS-4. The TDSA was operated in splitless mode, and used a temperature program for desorption of 20°C (hold for 0.5 min), then a 60° C.min^{-1} ramp to 270°C (final hold of 5 min). Temperature of the transfer line was set at 280°C. The CIS-4 was cooled with liquid nitrogen to −80°C. After desorption and cryotrapping, the CIS-4 was heated at 12°C.sec^{-1} to 280°C, with a hold time of 10 min. The CIS-4 inlet was operated in the solvent vent mode, with a vent pressure of 9.0 psi, a vent flow of 50 ml. min^{-1}, and a purge flow of 50 ml.min^{-1}. The temperature program for the GC oven was 40°C for 5 min, then increased to 200°C at 2°C.min^{-1} (hold time 10 min). The carrier gas had a constant flow of 1.0 ml.min^{-1}.

Quantitative Comparisons of Compound Levels Peak areas (PAs) were integrated from post-run selected ion chromatograms and divided by the peak area of the embedded internal standard (7-tridecanone) from the ion chromatogram for *m/z* 113 in the corresponding run (normalized peak area, NPA). For 2-ketones, *m/z* 58, phenol and 2-pyrrolecarboxaldehyde *m/z* 94, linear carboxylic acids *m/z* 73, linear alcohols *m/z* 55, and for acetamidofurans *m/z* 125, were used.

N-Acetylglucosamine-Derived Compounds and Tests Two unusual compounds, 3-acetamidofuran (3-AF) and 3-acetamido-5-acetylfuran (3-A-5AcF), which had been reported previously as thermal degradation products of N-acetylglucosamine in the ratio 2.5:1 (Franich and Goodin, 1984; Chen et al., 1998), were identified in our samples. In order to verify whether these compounds were thermal degradation or metabolic products, we conducted an experiment to degrade N-acetylglucosamine under the same conditions that the feline samples were collected and analyzed. Water solutions of N-acetyl glucosamine (8 μg/μl) were acidified with acetic acid, to mimic the presence of organic acids on the feline hair, and allowed to stand for 15 min, 24, 48, or 72 hr. An aliquot of 300 μl of the acidified solution was placed on aluminum foil and rolled over repeatedly with the roller pin device, in similar fashion as to how the facial samples were collected.

Synthetic 3-AF was used as a behavioral test compound. We tested unneutered males and unspayed females, to ensure neutering did not affect the response to the compound, on 7/8/2009, between 9 am and 12 pm. The individuals tested included three lions in two separate enclosures, one vasectomized male with a female, and one female alone, four tiger males, all housed alone, two leopard males, housed alone, and two cougar males, housed alone. A control solution of 10% glycerine in water and a test solution of 10^{-5} M 3-AF in 10% glycerine in water, were swabbed on the upper half of 25.4 cm hard plastic Boomer Balls ™, which were then placed within 2 m of each other in the center of an enclosure. When the animals were released back into the enclosure, positive responses to the solutions, including sniffing, flehmen, licking, rubbing, and urinating, were recorded for 5 min after initial contact. After that, the balls were usually moved as they were contaminated with other substances within the enclosure.

Results

Preliminary Scent Tests We observed a total of 54 positive responses to cardboard scent squares presented to lions. There were more responses ($X_1^2 = 7.4$, P=0.006) to head samples than to the control, but no difference ($X_1^2 = 0.4$, P= 0.54) in numbers of responses to cheek samples and

controls. Tigers showed 110 positive responses to the squares. There were more responses to head ($X_1^2 = 14.5$, P=0.001) and cheek samples ($X_1^2 = 16.0$, P=0.001) than to the control (Fig. 1). Forty positive responses by leopards showed no preference for either head ($X_1^2 = 0.92$, P=0.34) or cheek samples ($X_1^2 = 0.15$, P=0.70) over controls. Thirteen positive responses from cougars showed no differences between either head samples ($X_1^2 = 1.0$, P=0.32) and controls, or cheek samples ($X_1^2 = 0.4$, P=0.53) and controls. No preferences by leopards and cougars could indicate that the compounds deposited on the cardboard squares were more volatile and possibly lost during storage, or that facial compounds are not used as chemical signals in these species. Regardless, identifying facial compounds for comparison was deemed important.

Chemical Analysis of the Facial Surface Samples Reproducibility of the surface sampling analysis has been established previously at 6–10%, RSD (relative standard deviation) for different volatile compound classes (Soini et al., 2006). In this study, the repeatability for the embedded internal standard peak area was 8.6% (RSD, N=17).

In the samples, 100 volatile organic compounds were identified through matches with authentic compounds, or tentatively identified through spectral searches. Table 3 summarizes identified or tentatively identified compounds,

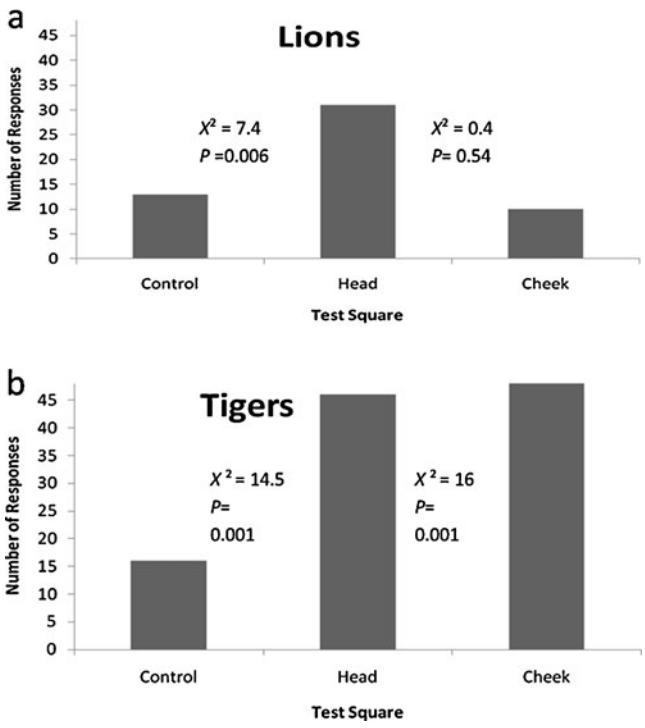

Fig. 1 Numbers of positive responses (including rubbing, spraying, flehmen, licking, and sniffing) to scent (from control, head or cheek of conspecific felines) deposited on cardboard squares for (**a**) lions and (**b**) tigers

organized by compound class. Compound identities were verified by authentic standards (59), except those (41) marked with an asterisk (Table 3), which are called "tentatively identified compounds", because a reference standard was not available for final verification. For tentative identification, NIST reference library match and in-house retention index and mass fragmentation interpretation databases were used. The same compounds were found in both forehead and cheek samples, but the cheek samples generally contained lower amounts (10–80% less, data not shown). The same compounds were found in all four species, although some compound levels varied substantially among species. In general, compound levels in lions and tigers were higher than in leopards and cougars, and may reflect differences seen among behavioral responses. Figure 2 illustrates the total ion chromatogram differences among lions, tigers, leopards, and cougars.

We found 41 compounds, including carboxylic acids, aldehydes, alcohols, ketones, amides, hydrocarbons, and aromatic compounds, that had been identified previously in both feline marking fluid compounds (a mixture of a lipid-based substance from anal sac mixed with urine), urine, and lion manes (Table 3). We also found several compounds not previously reported in feline studies. These included heterocyclic nitrogen and oxygen compounds, such as pyrroles, pyrrolidines, pyrrolidones, and furans. Acetamidofuran and its derivatives were also found. Previously, we found 3-AF on the hair of domestic cats at a much lower concentration (unpublished results). Selected chemical structures for these compounds are shown in Fig. 3.

Compound Level Differences Among Species, Individuals and Sexes Quantitative differences were seen among species for some compounds. 2-Pentadecanone and 2-heptadecanone levels in tiger foreheads were consistently high (peak area ratios 0.2–0.9), while the other species showed only trace levels (peak area ratios<0.002) of these compounds (Fig. 2, peaks 15 and 18). Species level differences for three linear carboxylic acids (tetradecanoic, hexadecanoic, and octadecanoic acids) also were found, with lions and tigers showing substantially higher levels than leopards and cougars (Fig. 2, peaks 16, 20, and 24).

A large amount of individual variation was seen within each species regarding the above compounds. For example, tiger 2-pentadecanone and 2-heptadecanone levels differed four-fold among some individuals (Fig. 4). There were also some interesting differences between the sexes (see Fig. 4, e.g., Anna and Tony). Higher levels of carboxylic acids and acetamidofurans were found in cougar females than in males, but the opposite was found for leopards. There were mixed results with lions and tigers, and there seemed to be no general trend among species. With small sample sizes of

Table 3 Volatile compounds identified from four feline species

	Compound	Retention time (min)	Reported in felines
MW	**Carboxylic acids**		
60	Acetic acid	3.63	[b]tiger, [c]leopard, [i]lion
102	3-Methylbutanoic acid (isovaleric acid)	9.46	[i]lion
116	Hexanoic acid	18.61	[b]tiger, [c]leopard
130	Heptanoic acid	25.10	[b]tiger, [c]leopard
144	Octanoic acid	32.14	[b]tiger, [c]leopard
158	Nonanoic acid	38.48	[b,d]tiger, [c]leopard
172	Decanoic acid	44.76	[b,d]tiger
200	Dodecanoic acid	56.96	[b,d]tiger
214	Tridecanoic acid	62.12	[e]lion
228	Tetradecanoic acid (myristic acid)	67.25	[b,f]tiger, [e]lion, [g]cheetah
242	Pentadecanoic acid (branched)[a]	71.13	
242	Pentadecanoic acid	72.31	[b]tiger, [e]lion
254	Palmitoleic acid ((Z)-9-hexadecenoic acid)	76.12	[b,f]tiger, [g]cheetah
256	Hexadecanoic acid (palmitic acid)	77.28	[b,f]tiger, [e]lion, [j]bobcat
270	Heptadecanoic acid	82.16	[b]tiger, [e]lion
280	Linoleic acid (9,12-octadecadienoic acid)	85.25	
282	Oleic acid ((Z)-octadecenoic acid)	85.33	[b]tiger, [e]lion, [j]bobcat
284	Octadecanoic acid (stearic acid)	86.54	[b,f]tiger, [e]lion
MW	**Aldehydes**		
96	Furfural[a]	8.33	
114	Heptanal	12.17	[b]tiger, [h]lion
106	Benzaldehyde	15.76	[b]tiger, [g]cheetah
110	5-Methylfurfural[a]	15.76	
128	Octanal	18.91	[b]tiger, [g]cheetah, [h]lion
95	2-Pyrrolecarboxaldehyde[a]	19.62	
126	2-Octenal	22.73	[b]tiger, [h]lion
124	4-Ethyl-2-furaldehyde[a]	24.42	
142	Nonanal	26.20	[b]tiger, [g]cheetah, [h]lion
140	2-Nonenal	30.05	
112	Decanal	33.41	[b]tiger, [g]cheetah
154	2-Decenal	37.19	
168	2-Undecenal	46.41	
MW	**Alcohols**		
102	3-Hexanol	7.21	
98	Furfuryl alcohol[a]	9.46	[b]tiger
90	2-(2-Ethoxyethyl)-ethanol[a]	18.81	
130	2-Ethyl-1-hexanol	20.74	
144	1-Octanol	23.80	[b]tiger
158	1-Decanol	38.01	
214	1-Tetradecanol	62.69	[b,f]tiger
242	1-Hexadecanol	73.32	[b,f]tiger
270	1-Octadecanol	83.04	[b,f]tiger
284	1-Nonadecanol	87.79	
298	1-Eicosanol (branched)[a]	90.02	
386	Cholesterol	92.13	
298	1-Eicosanol[a]	94.00	[f]tiger
MW	**Ketones**		
74	Hydroxyacetone[a]	4.22	

Table 3 (continued)

	Compound	Retention time (min)	Reported in felines
116	4-Hydroxy-4-methyl-2-pentanone[a]	9.30	
84	2(5H)-Furanone	12.66	
98	Cyclohexanone	13.69	[g]cheetah, [h]lion
126	6-Methyl-5-hepten-2-one	17.59	
120	Acetophenone	23.00	[b]tiger, [g]cheetah
156	2-Decanone	32.37	[b]tiger
194	Geranylacetone	49.18	[b]tiger
226	2-Pentadecanone	63.75	[b]tiger
254	2-Heptadecanone	74.24	[b]tiger
282	2-Nonadecanone	83.75	
MW	**Amides**		
59	Acetamide	6.62	[b]tiger
101	N,N-Diethylformamide	14.35	
129	Heptanamide[a]	26.91	
283	Stearamide (octadecanamide)[a]	86.91	[j]bobcat
MW	**Heterocyclic nitrogen ring compounds**		
71	Pyrrolidine[a]	13.37	
109	N-Acetylpyrrole[a]	15.37	
113	2-Acetylpyrrolidine[a]	16.19	
125	Z-N-Propenylpyrrolidinone[a]	18.41	
125	E-N-Propenylpyrrolidinone[a]	19.05	
141	An alkylpyrrolidine[a]	19.59	
126	2-(3-Pentenyl)imidazole[a]	23.56	
153	N-Pentenylpyrrolidinone[a]	27.99	
123	An acetylmethylpyrrole[a]	29.88	
113	2-Propylpyrrolidine[a]	29.89	
137	2-Methyl-5-propionylpyrrole[a]	33.27	
MW	**Heterocyclic oxygen ring compounds**		
128	γ-Heptanolactone[a]	28.57	
125	3-Acetamidofuran	34.86	
139	4-Acetamido-2-methylfuran[a]	40.68	
125	4-Amino-2-acetylfuran[a]	41.18	
141	3-Acetamido-4-hydroxyfuran[a]	42.09	
167	3-Acetamido-5-acetylfuran	59.96	
MW	**Hydrocarbons**		
142	Decane	18.75	[b]tiger, [g]cheetah, [h]lion
170	Dodecane	33.02	
182	1-Tridecene	39.32	
184	Tridecane	39.88	
196	1-Tetradecene	45.88	
198	Tetradecane	46.42	[b]tiger
210	1-Pentadecene	52.10	
212	Pentadecane	52.58	
410	Squalene[a]	96.40	[b]tiger
MW	**Esters**		
126	A methyl imidazolecarboxylate[a]	24.09	
250	2-Ethyl-1-hexyl salicylate[a]	70.70	
262	3,3,5-Trimethylcyclohexyl salicylate [a]	72.82	
290	2-Ethyl-1-hexyl x-methoxycinnamate[a]	85.99	

Table 3 (continued)

	Compound	Retention time (min)	Reported in felines
290	2-Ethyl-1-hexyl y-methoxycinnamate[a]	95.19	
MW	**Aromatic compounds**		
94	Phenol	17.33	[b]tiger, [g]cheetah, [h]lion
134	*p*-Cymene	20.29	
117	Phenylacetonitrile[a]	28.24	
138	2-Phenoxyethanol[a]	33.90	
135	Benzothiazole	34.00	
131	Benzenepropanenitrile[a]	35.22	
117	Indole	38.77	[j]bobcat
151	An acetamidophenol[a]	46.88	
167	An acetamidoresorcinol[a]	52.29	
167	An acetamidoresorcinol[a]	53.61	
228	A benzoyl-methoxyphenol[a]	79.42	

[a] Tentative identification based on NIST library match and internal retention index and mass fragmentation databases

[b] Burger et al. 2008. Tiger marking fluid (MF)

[c] Poddar-Sarkar and Brahmachary 2004. Leopard marking fluid (MF)

[d] Poddar-Sarkar et al. 2004. Tiger marking fluid (MF)

[e] Poddar-Sarkar et al. 2007. Lion mane

[f] Poddar-Sarkar 1996. Tiger marking fluid lipids.

[g] Burger et al. 2006. Cheetah urine

[h] Anderson and Vulpius 1999. Lion urine

[i] Albone and Eglington 1974. Lion anal sac

[j] Mattina et al. 1991. Bobcat urine

only three individuals of each sex, non-parametric statistical analysis showed no differences.

N-Acetylglucosamine-Derived Compounds and Tests Acetamidofuran compounds, which have been reported as biodegradation and thermal degradation products from chitin and its monomer N-acetylglucosamine (Stankiewicz et al., 1996), were considered as possible sources of scent cues, because they were present in facial samples from all investigated species. In the N-acetylglucosamine thermal degradation tests, measurable levels of 3-AF were obtained only after 24 and 48 hr of treatment, while 3-A-5AcF appeared as a trace signal after 72 hr treatment with acetic acid. The ratios of 3-AF to 3-A-5AcF in feline forehead samples varied from 2.2 to 8.9:1 for lions, 3.8–7.3:1 for tigers, 5.1–32.5:1 for cougars, and 3.3–5.8:1 for leopards, which deviate from the ratio of 2.5:1 reported for pure thermal degradation (Franich and Goodin, 1984). This suggests that 3-AF and 3-A-5AcF on feline foreheads are derived from metabolic production. Figure 5 illustrates the average normalized peak areas for 3-AF and 3-A-5AcF in the feline species.

In preliminary behavioral tests with 3-AF, we found no differences in positive responses to the control vs. test Boomer Ball™ for lions (23 responses; $X_1^2=1.08$, $P=0.30$), tigers (57 responses; $X_1^2=0.16$, $P=0.69$), and leopards (19 responses; $X_1^2=0.06$, $P=0.81$). Cougars only responded four times; this sample size was too small for statistical analysis. Behavioral tests with 3-A-5AcF were not conducted.

Discussion

Behavioral Responses to Scents Head- and cheek-rubbing behavior of felines is generally difficult to study in the wild, because most feline species are solitary and nocturnal. In the wild, rubbing on objects has been documented at marking sites for tigers (Smith et al., 1989) and leopards (Bothma and le Riche, 1995), and lions are known to rub other individuals and objects (Schaller, 1972). In several species of small felines in captivity, head- and cheek-rubbing on objects has been recorded, but the rubbing was not directed at individuals (Mellen, 1993). In our preliminary study, head- and cheek-rubbing could be observed systematically in captive large felines. Preliminary tests with cardboard scent squares indicated that volatile compounds deposited by rubbing elicited responses from lions and tigers. Leopard

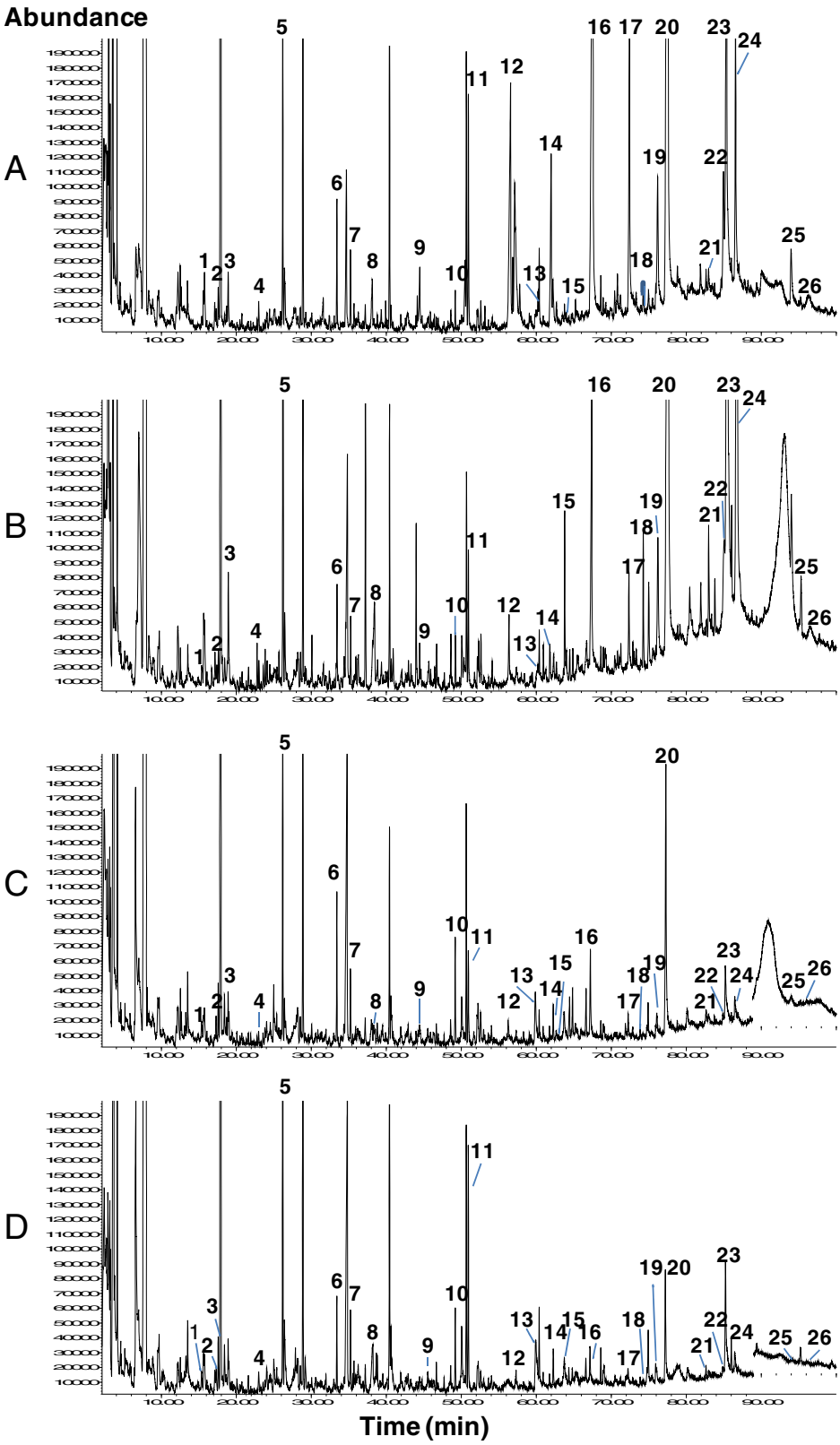

Fig. 2 Total ion chromatograms of compounds from male **a**: lion, **b**: tiger, **c**: leopard, **d**: cougar. Selected compounds indicated in the graphs are 1: benzaldehyde, 2: phenol, 3: octanal, 4: acetophenone, 5: nonanal, 6: decanal, 7: 3-acetamidofuran (3-AF), 8: nonanoic acid, 9: decanoic acid, 10: geranylacetone, 11: undecanoic acid, 12: dodecanoic acid, 13: 3-acetamido-5-acetylfuran (3-A-5AcF), 14: tridecanoic acid, 15: 2-pentadecanone, 16: tetradecanoic acid, 17: pentadecanoic acid, 18: 2-heptadecanone, 19: palmitoleic acid, 20: hexadecanoic acid, 21: 1-octadecanol, 22: linoleic acid, 23: oleic acid, 24: octadecanoic acid, 25: 1-eicosanol, 26: squalene

and cougars rubbed objects, but did not respond to rubbings, which may indicate that urine and other secretions may be a more important volatile compound source for these species, or that important volatiles may have evaporated from samples before they were presented to the cats. The compound 3-AF did not elicit any behavioral response from any of the

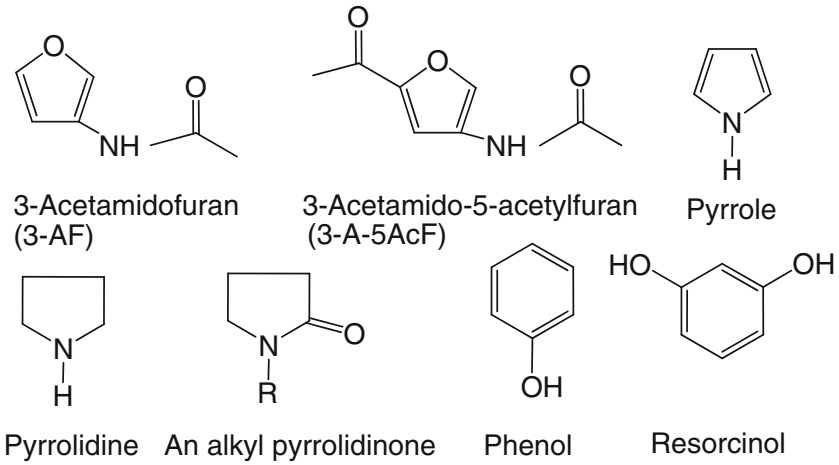

Fig. 3 Selected chemicals found on the foreheads of large felines

species. Future tests with 3-AF and other compounds, in different concentrations and blends, need to be conducted to determine what substances are important to each of the species. Also, other scent presentation methods need to be tested to rule out possible background scent disturbance from the Boomer Balls™.

Facial Volatile Compounds Previous authors suggested that volatile compounds may act as important scent cues of health and physical condition. Carboxylic acids identified in our facial samples are of particular interest. These acids are commonly found in mammalian samples, including human axillary secretions (Natsch et al., 2006; Penn et al., 2007). They have been identified in feline urine, marking fluids, and lion manes (Poddarr-Sarkar et al., 1994, 2007; Burger et al., 2008). These short-chain acids are products of fatty acid metabolism and bacterial activity (Natsch et al., 2006), and may be indicators of health and metabolic condition. Lipid metabolism differences have been documented for tiger, lion, cougar, and cheetah urine in a zoo environment (Asa, 1993). In our study, the higher levels of carboxylic acids found in tigers and lions, compared to the other species, may reflect total lipid metabolism differences among the species, as well as differences among individuals within a species. Our findings of higher levels of linear carboxylic acids in tigers and lions in facial scent samples are in accord with the previous findings in urine.

Methylketones (2-ketones) also are related to fatty acid metabolism and are enzymatic conversion products from linear carboxylic acids (Pannell and Olson, 1991). Other smaller and more volatile 2-ketones, such as 2-octanone,

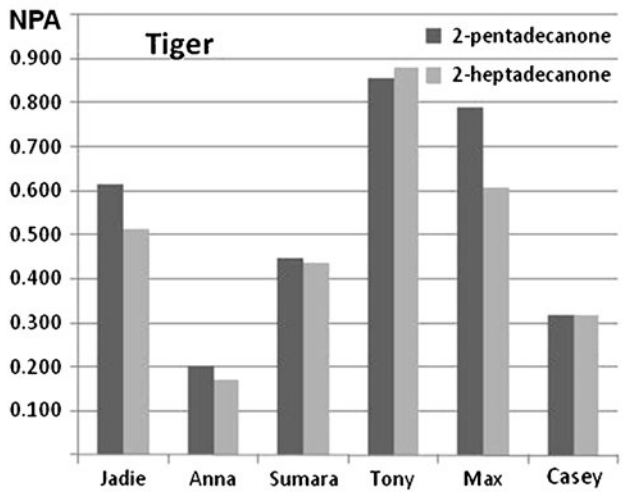

Fig. 4 Normalized peak areas (NPA) from post-run selected peak areas of *m/z* 58 for 2-pentadecanone (Rt 63.75 min) and 2-heptadecanone (Rt 74.24 min) in tiger forehead samples

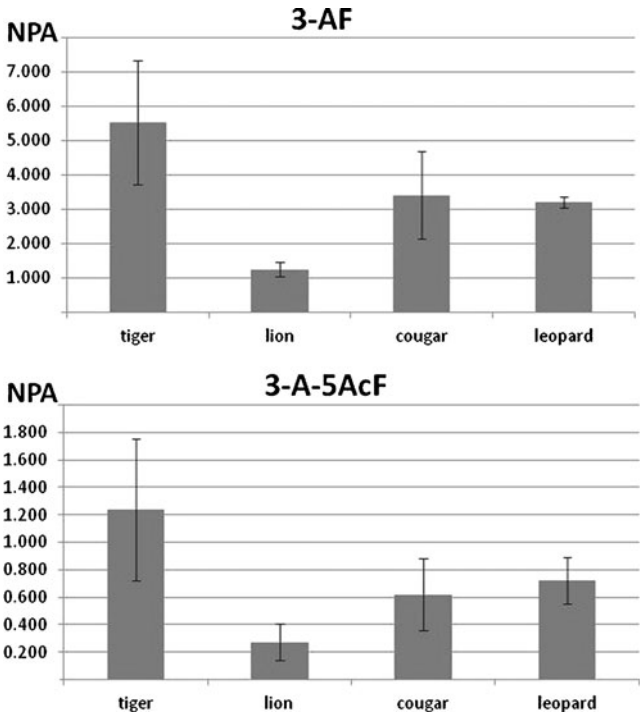

Fig. 5 Averages (±SEM) for normalized peak areas (NPA) for 3-acetamidofuran (3-AF) and 3-acetamido-5-acetylfuran (3-A-5AcF) in males and females in four feline species, measured from post-run *m/z* 125 peak areas (Rt 34.86 min)

2-nonanone, and 2-undecanone, also have been reported in cheetah urine (Burger et al., 2006), while 2-pentanone and 2-heptanone were found in lion urine (Anderson and Vulpius, 1999). In our study, high levels of 2-pentadecanone and 2-heptadecanone were found in captive tigers. These are the same compounds found in territorial marking fluid of Bengal tigers (Burger et al., 2008), and may function as indicators of tiger-specific fatty acid metabolism and in chemical signaling.

Heterocyclic oxygen and nitrogen compounds have not been identified previously in felines. Furans have been reported in a wide range of mammalian samples. Rat urine (Holland et al., 1983) and rat preputial gland secretions (Pohorecky et al., 2008), as well as wolf urine (Raymer et al., 1986) and anal-sac secretions (Raymer et al., 1985), contained furan alkyl and acetyl derivatives, but not the amino derivatives found in this study. N-Acetylpyrrole, identified here, has been reported in human skin (Penn et al., 2007) and rat urine (Holland et al., 1983). Pyrrole levels in urine and skin may reveal individual health, as increased pyrrole levels have been reported in human urine after stress (Jackson et al., 1997).

Two aromatic compounds, phenol and indole, have been identified in the urine of several felines (Mattina et al., 1991; Anderson and Vulpius, 1999; Burger et al., 2006, 2008). Indole is often identified in carnivore samples, but also has been found in Siberian Hamster urine (Soini et al., 2005).

N-Acetylglucosamine was originally identified as a monomer of chitin, which is part of the support structures of fungi, parasites, and bacteria (Stankiewicz et al., 1996). Recently, mammalian acidic chitin-degrading chitinase has been found in humans and mice (Renkema et al., 1995; Boot et al., 2001). The authors suggested that chitin-degrading metabolic pathways might be connected to mammalian pathogen resistance mechanisms.

In summary, this comparative study of four large felines has identified a list of possible volatile compounds associated with their behavior. In prior chemical signaling research, apart from the few compounds identified in lion manes and some indication of sexual differences in lion urine compounds, no reports exist linking such volatile compounds to behavior. More work is needed in identifying individual compounds and determining which of those compounds may induce important behavioral cues.

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(*R*)-Desmolactone, A Female-produced Sex Pheromone Component of the Cerambycid Beetle *Desmocerus californicus californicus* (subfamily Lepturinae)

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Abstract We report the identification, synthesis, and field bioassays of a female-produced sex attractant pheromone for the cerambycid beetle *Desmocerus californicus californicus* Horn. Headspace volatiles from females contained a sex-specific compound, (*R*)-desmolactone [(4*R*,9*Z*)-hexadec-9-en-4-olide], which elicited strong responses from the antennae of adult males in coupled gas chromatography-electroantennogram analyses. Short syntheses of both enantiomers were developed from commercial chiral synthons. In field bioassays, significant numbers of males were collected in traps baited with (*R*)-desmolactone, whereas the (*S*)-enantiomer attracted no males. The racemate was less attractive than the pure (*R*)-enantiomer, indicating some degree of antagonism by the unnatural enantiomer. This compound is the first example of a new structural class of cerambycid pheromones, and is the second pheromone identified for a species in the subfamily Lepturinae.

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Introduction

In recent years, volatile sex or aggregation pheromones have been identified for a number of species in the cerambycid beetle subfamilies Cerambycinae, Lamiinae, Prioninae, and Spondylidinae, suggesting that pheromones play an important role in mate location in these groups (Silk et al., 2007; Millar et al., 2009; Ray et al., 2009; Fonseca et al., 2010; Pajares et al., 2010; Barbour et al., 2011; Teale et al., 2011; Paschen et al., unpublished). However, until recently, no pheromones had been reported for species in the subfamily Lepturinae (*sensu* Napp, 1994). It has been hypothesized that lepturines do not use volatile sex or aggregation pheromones, relying instead on attraction of both sexes to the adult food plant (Hanks, 1999). However, our recent identification of a female-produced sex pheromone for the lepturine species *Ortholeptura valida* (LeConte), suggests that volatile pheromones may indeed play a role in mate location in at least some lepturine species (Ray et al., 2011).

The subfamily Lepturinae comprises a small group of cerambycid species that are primarily Holarctic in distribution (Linsley, 1959; Švácha and Danilevsky, 1986). Lepturines commonly are referred to as "flower longhorns" because adults of many species feed on pollen or nectar (Linsley, 1961). Larvae of most lepturine species develop in decaying wood (Swaine and Hopping, 1928; Michelsen, 1963; Linsley and Chemsak, 1972). The behavior of adults is not well understood, and little is known of mate location or recognition strategies (e.g., Linsley and Chemsak, 1972;

Hanks, 1999). In some species, short-range mate recognition is likely mediated by contact pheromones (Michelsen, 1963).

The lepturine species *Desmocerus c. californicus* Horn is endemic to coastal California, where it occurs from Trinity County to San Diego County (Halstead and Oldham, 2000). Larvae of *D. c. californicus* develop in the stems of living *Sambucus nigra* L. ssp. *canadensis* (L.) Bolli shrubs (Caprifoliaceae) (Burke, 1921; Linsley and Chemsak, 1972). Adults emerge from late March to May and are diurnal, feeding on the leaves and flowers of the host plant (Davis and Comstock, 1924; Linsley and Chemsak, 1972; Solomon, 1995). Adults mate on the host (Burke, 1921).

As part of a long-term project to elucidate the pheromone chemistry and pheromone-mediated biology of cerambycids, we report here the identification, synthesis, and field bioassays of a female-produced sex attractant pheromone from *D. c. californicus*. The active compound, (4*R*,9*Z*)-hexadec-9-en-4-olide, represents the first example of a new structural class of pheromones within the Cerambycidae.

Methods and Materials

Source of Beetles We collected pupae of *D. c. californicus* on 19 April 2010 from healthy *S. nigra* ssp. *canadensis* growing in an elderberry savanna along East San Bernardino Avenue in Redlands, CA (San Bernardino Co., CA, USA). Infested trees were identified by the expelled frass at the base of the trunks (Solomon, 1995). Pupae were cut from pupal chambers in the root crown and trunks of infested trees. Individual pupae were placed into artificial pupation chambers (newsprint tubes, ~7 cm long, ~2 cm diam; see Ray et al., 2011), the tube ends were plugged with tissue paper, and the tube was misted with water. Tubes were stored together in a 4 l plastic bag at room temperature. Tubes were examined 2–3 times per week to determine if pupae had developed into adults. Adults began emerging between 2 and 4 May 2010. Teneral adults were returned to their tubes, the ends were plugged with paper, and the tubes were held at 4°C until all other adults emerged. Sixteen hours prior to starting headspace collections, unmated, adult *D. c. californicus* were placed into individual 20 ml vials and brought to room temperature.

Collection of Volatiles We conducted initial aerations of groups of male or female beetles in the laboratory under ambient conditions (ca. 26°C with variable humidity) between 5 and 14 May 2010. Headspace odors were collected from either 2 males or 4 females in glass chambers (13 cm diam× 40 cm long), with light provided by a west-facing window and indoor fluorescent lighting. Stems of *S. nigra* ssp. *canadensis* (with both inflorescences and leaves) in vials of water were provided as a food source. As a control, headspace odors were also collected from *S. nigra* ssp. *canadensis* stems in water, without beetles. Airflow of ca.1 to 2 l/min was provided by a flow meter-controlled vacuum source with charcoal-filtered (6–14 mesh, Fisher Scientific, Pittsburgh, PA, USA) room air being pulled through the chamber. Collectors consisted of 6 mm outer diam glass tubes with ca. 1.5–2 cm long beds of thermally desorbed 50–200 mesh activated charcoal (Fisher Scientific, Pittsburgh, PA, USA) held in place by Soxhlet extracted (pentane, then ethyl acetate) glass wool plugs. Collectors were connected to ground glass fittings and the vacuum source by using 1/2 to 1/4 inch and 1/4 to 1/4 inch Swagelok® unions, respectively, with Teflon ferrules (Swagelok®, San Diego Valve and Fitting Co., San Diego CA, USA). Aerations were conducted for 2 to 3 d, after which the collectors were eluted with 3 rinses of dichloromethane to a final volume of ca. 1 ml. Extracts were stored at ~−20°C until needed. Subsequent aerations were conducted on individual female insects in an environmentally controlled room (ca 26°C, room fluorescent lights on a 15:9 h L:D cycle, humidity not regulated or monitored) in 3 cm inner diam× 25 cm long chambers with a single *S. nigra* ssp. *canadensis* leaf in water. Leaves and collectors were changed every other day. Airflow (300 ml/min) was provided by house vacuum, and collectors were extracted as described above. As controls, *S. nigra* ssp. *canadensis* leaves that had not been fed upon were aerated, as well as leaves that had been fed upon by males.

Analyses of Extracts Coupled gas chromatography-electroantennogram detection (GC-EAD) analyses were performed with DB-5 and DB-Wax columns (both 30 m× 0.25 mm i.d., 0.25 μm film; J&W Scientific, Folsom, CA, USA). Helium was used as carrier and makeup gas. The GC oven was programmed from 50°C for 1 min, 10°C per min to 275°C for DB-5, and 250°C for DB-Wax, hold for 60 min. Extracts (1 μl aliquots) were analyzed in splitless mode. The effluent from the columns was split equally between the GC detector and the EAD. The portion directed to the EAD was diluted in a humidified air stream that was directed over the antennal preparation, which consisted of the excised terminal 4–5 antennal segments of a male beetle, with the distal tip excised with a razor blade, placed between two saline-filled glass capillary electrodes (7.5 g NaCl, 0.21 g $CaCl_2$, 0.35 g KCl, and 0.20 g $NaHCO_3$ in 1 l Milli-Q® purified water). The glass electrodes were fitted with 0.2 mm diam gold wires that connected to a custom built EAD amplifier, with the amplifier gain at 100X amplification. A single antennal preparation was used for as many as six runs.

GC-MS analyses were carried out with an Agilent 6890 N GC interfaced to a 5975 C mass selective detector (Agilent, Santa Clara, CA, USA). The GC was fitted with an HP5-MS column (30 m×0.25 mm i.d., 0.25 μm film), and

the same temperature program and injection conditions were used as described above. Retention index (RI) values were calculated for unknowns and standards relative to blends of straight-chain hydrocarbons. For increased precision, RI values on the DB-Wax column were obtained using a GC oven program rate of 5 rather than 10°C/min.

To determine the absolute configuration of the pheromone, aliquots of extracts were analyzed on a chiral stationary phase ß-Dex 225 column (25% 2,3-di-O-acetyl-6-O-TBDMS-ß-cyclodextrin in SPB-20, 30 m×0.25 mm ID, 0.25 micron film thickness; Supelco, Bellefonte, PA, USA) with an oven temp of 185°C. The enantiomers did not resolve on a Cyclodex B® column (10.5% ß-cyclodextrin in DB-1701; J&W Scientific, Folsom, CA, USA).

Derivatization Reactions An aliquot of an extract containing the female-specific compound was reduced by addition of ~1 mg 5% Pd on carbon and stirring for 1 h under a hydrogen atmosphere. The catalyst was removed by filtration through a 5 mm bed of Celite in the tip of a Pasteur pipette plugged with glass wool.

A second aliquot of the extract was evaporated to dryness, then taken up in 0.1 ml hexane, and treated with 5 µl of dimethyldisulphide and 1 drop of a solution of iodine in ether (10 mg/ml) in a glass vial with a Teflon cap-liner. The solution was warmed to 50º overnight, then diluted with 0.5 ml pentane and extracted with 1 M $Na_2S_2O_3$ solution (100 µl). The colorless organic layer was dried over anhydrous Na_2SO_4, concentrated under a stream of nitrogen, and analyzed by GC-MS on the HP5-MS column, with injector and oven final temperatures of 280°C.

To provide samples of both the (*E*)- and (*Z*)-isomers of desmolactone, (*S*)-desmolactone (10 mg, see below) and thiophenol (5 µl) were combined in a conical bottomed glass vial with a Teflon-lined screw cap, and the mixture was heated in an oil bath at 100°C for 30 min. The mixture then was cooled to room temperature, diluted with hexane, and vortexed with aqueous 1 M NaOH. The hexane layer was removed and dried over anhydrous Na_2SO_4. A portion of the resulting solution (~1 mg of the *E/Z* mixture) was treated with *m*-chloroperbenzoic acid (100 µl of a 20 mg per ml solution in CH_2Cl_2, 2 mg) at room temperature for 3 h. The solution then was concentrated under a stream of nitrogen, and the residue was taken up in hexane and vortexed with aqueous 1 M NaOH. The hexane layer was separated, dried over anhydrous Na_2SO_4, and analyzed by GC-MS on a DB-5MS column as described above. A sample of pure (*S*)-desmolactone (see below for synthesis) and an aliquot of an aeration extract from female *D. c. californicus* were epoxidized and analyzed using the same conditions.

Synthesis of (R)- and (S)-Desmolactones (Fig. 1) ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian INOVA-400 spectrometer (Palo Alto, CA, USA) (400 and 100.5 MHz, respectively), as $CDCl_3$ solutions. High resolution mass spectra were obtained by direct injection on a Waters GCTOF instrument (Waters Corp., Milford, MA, USA). Optical rotations were measured in CH_2Cl_2 on a Perkin-Elmer 241 polarimeter (Na and Hg lamps) (Perkin Elmer, Waltham, MA, USA) in a 1 ml water jacketed cell (10 cm path length).

1-Chloroundec-4-yne (**2**) Butyllithium (2.87 M in hexanes) was added dropwise to a solution of ~50 mg triphenylmethane indicator in 400 ml dry THF under Ar until a red color persisted indicating that all water and dissolved oxygen had been consumed. 1-Octyne **1** (16.1 ml, 110 mmol) was added, the solution was cooled in an ice-bath, and BuLi (38 ml of 2.87 M, 110 mmol) was added dropwise until a red color persisted again, followed by NaI (1.5 g) and dropwise addition of 1-bromo-3-chloropropane (23.6 g, 150 mmol). When the addition was complete, the solution was warmed to 60°C and stirred overnight, then warmed to 70°C and stirred overnight again, producing an orange suspension. After cooling, the mixture was quenched by addition of ice-water, and extracted twice with hexane. The combined hexane layers were washed with brine, concentrated, and purified by Kugelrohr distillation (bp~80°C at 6 mm Hg), yielding 19.94 g (97%) of the known chloride (Lie Ken Jie, 1975) as a malodorous clear liquid. ^{1}H NMR: δ 3.66 (t, 2H, J=6.4 Hz), 2.35 (tt, 2H, J=6.8, 2.4 Hz), 2.15 (tt, 2H, J=7.2, 2.4 Hz), 1.94 (quint, 2H, J=6.4 Hz), 1.48 (quint, 2H, J=6.4 Hz), 1.42-1.24 (m, 6H), 0.90 (t, 3H, J= 6.8 Hz). ^{13}C NMR: δ 81.6, 78.1, 44.0, 31.0, 31.5, 29.2, 28.7, 22.7, 18.9, 16.4, 14.2 ppm.

(Z)-1-Chloroundec-4-ene (**3**) $Ni(OAc)_2•4H_2O$ (4.98 g, 20 mmol) was dissolved in 200 ml EtOH, and the resulting green solution was degassed under Ar. A 1 M solution of $NaBH_4$ was prepared by dissolving 1.14 g $NaBH_4$ in 28 ml EtOH and 2 ml of aqueous 2 M NaOH, and the slightly cloudy solution was filtered. Twenty ml of the resulting clear solution were added to the vigorously stirred solution of $Ni(OAc)_2•4H_2O$ under argon over 2 min, giving a black suspension. This was stirred 10 min, then ethylene diamine (4 ml, 60 mmol) was added, the suspension was stirred again for 10 min, then the argon line with replaced with a balloon filled with H_2, and 18.7 g (100 mmol) of chloroalkyne **2** were added by syringe. The reduction was followed by GC, and was complete after 9 h. The reaction mixture was filtered through a plug of activated charcoal layered over Celite, rinsing the filter cake with EtOH. The violet colored filtrate was concentrated by rotary evaporation, then partitioned between 1 M HCl and hexane. The hexane layer was washed with saturated aqueous $NaHCO_3$ and brine, dried, concentrated, and purified by Kugelrohr distillation (bp ~80 at 7 mm Hg), giving 13.34 g (71%) of the known chloroalkene **3**

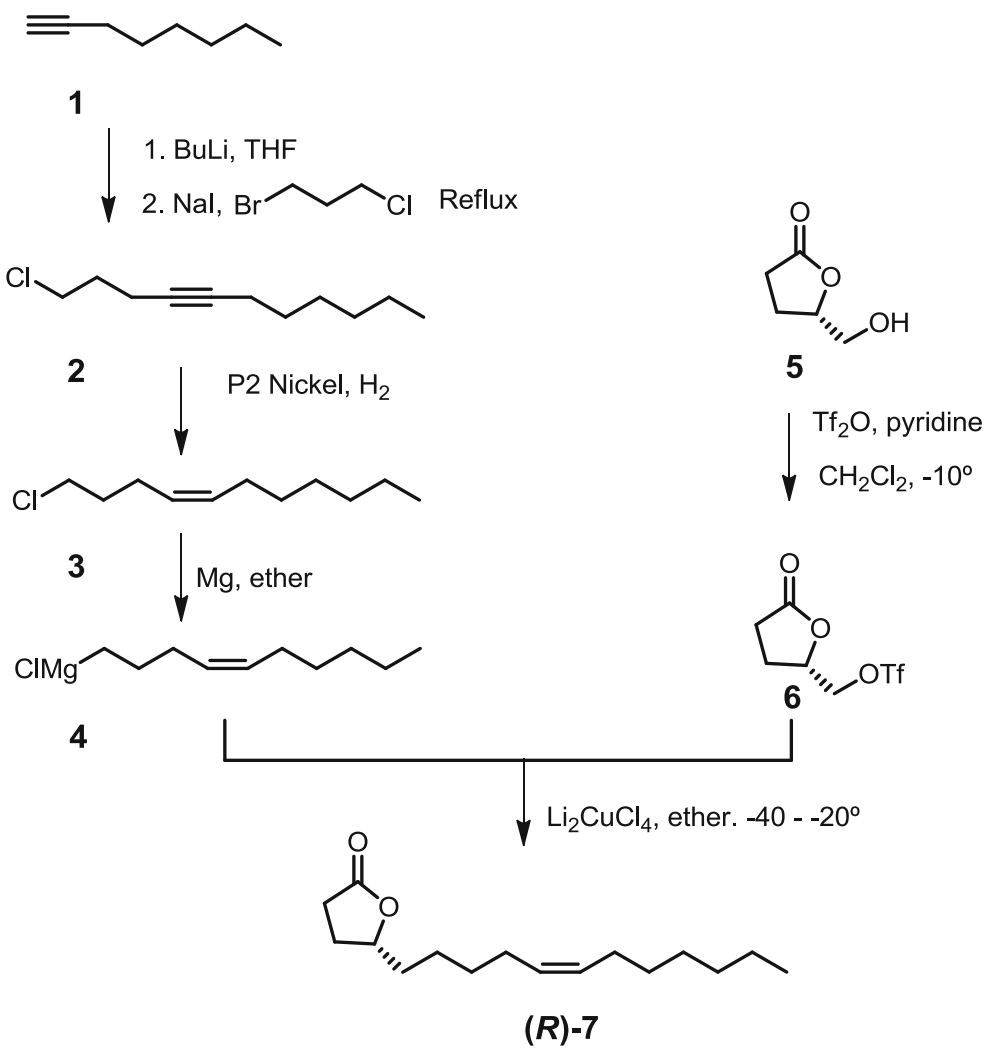

Fig. 1 Synthesis of (*R*)-desmolactone

(Fukumoto and Ooshima, 1994), contaminated with ~2% each of the *trans* isomer and saturated undecyl chloride. ^{1}H NMR: δ 5.45 (dtt, 1H, J=10.8, 7.2, 1.4 Hz), 5.32 (dtt, 1H, J=10.8, 7.2, 1.4 Hz), 3.55 (t, 2H, J=6.6 Hz), 2.20 (br quart 2H, J=7.2 Hz), 2.05 (br quart, 2H, J=6.8 Hz), 1.83 (quint, 2H, J=6.9 Hz), 1.39-1.23 (m, 8H), 0.90 (t, 3H, J=6.4 Hz). ^{13}C NMR: δ 131.9, 127.7, 44.7, 31.9, 29.8, 29.1, 27.4, 24.5, 22.8, 14.2 ppm.

(4R,7Z)-Hexadec-9-en-4-olide (**7**)

(a) A solution of (5*S*)-dihydro-5-(hydroxymethyl)-2(3H)-furanone **5** (2.5 g, 21.6 mmol; TCI America, Portland, OR, USA), pyridine (1.7 ml, 22 mmol), and CH_2Cl_2 (50 ml) was cooled to <−10°C in an acetone/ice bath, and trifluoromethanesulfonic anhydride (4.34 ml, 25.8 mmol) was added over 5 min. The mixture was stirred 1 h, letting it warm to ~0°C, at which time GC analysis indicated that the starting material had been consumed. The mixture was filtered through a 2 cm plug of silica gel that had been prewetted with CH_2Cl_2, and the silica gel was rinsed with an additional 150 ml of CH_2Cl_2. The clear, colorless solution was concentrated without heating, then pumped under vacuum for 10 min to remove traces of excess triflic anhydride. The resulting triflate **6**, obtained as a viscous oil (4.86 g, 91%), was used directly in the next step.

(b) Magnesium chips (1.08 g, 45 mmol) were ground briefly with a mortar and pestle to expose fresh metal, then added to a dry 3-neck flask flushed with argon. (*Z*)-1-Chloroundec-4-ene **3** (6.1 g, 32.5 mmol) was dissolved in 60 ml dry ether, and 15 ml of the solution were added to the flask, along with a few crystals of iodine. After the Grignard reaction had started, as evidenced by the warming of the solution, the remainder of the ether solution of the chloride was added in 5 ml aliquots over 2 h. When the addition was complete, the mixture was refluxed 1 h, then cooled to room temperature.

(c) Triflate **6** prepared in (a) above (3.47 g, 14 mmol) was dissolved in 60 ml ether in a dry flask flushed with argon, Li_2CuCl_4 was added (15 ml of 0.1 M THF solution; Aldrich Chem., Milwaukee, WI, USA), the solution was cooled to <−40°C, and the ether solution of the

Grignard reagent **4** prepared in (b) was added over 20 min, maintaining the temperature <−35°C. The solution then was allowed to warm to −20º, at which point TLC analysis indicated that all the triflate had been consumed. The reaction was quenched by addition of saturated aqueous NH_4Cl and extracted with ether. The ether layer was washed with water and brine, treated with decolorizing charcoal and anhydrous Na_2SO_4, filtered through a Celite plug, and concentrated. The crude product was purified by vacuum flash chromatography on silica gel, eluting with a stepwise gradient of hexane, 10% EtOAc in hexane, and 20% EtOAc in hexane, yielding 3.29 g (93%) of the desired lactone contaminated with ~2% of the saturated analog. Analysis on the ß-Dex 225 column indicated an ee of >95% (retention time 39.76 min). ^{1}H NMR: δ 5.36 (m, 2H), 4.49 (m, 1H), 2.54 (m, 2H), 2.32 (br sextet, 2H, J ~6.5 Hz), 2.03 (br. sextet, 2H, J=6.8 Hz), 1.9-1.25 (m, ~16H), 0.89 (t, 3H, J= 7.2 Hz). ^{13}C NMR: δ 177.3, 130.6, 129.3, 81.1, 35.7, 31.9, 29.9, 29.6, 29.1, 29.0, 28.2, 27.4, 27.1, 25.0, 22.8, 14.3 ppm. MS (EI, 70 eV) (*m/z*, abundance): 252 (10), 234 (1), 192 (7), 179 (4), 167 (5), 150 (15), 136 (32), 122 (21), 109 (28), 95 (54), 85 (48), 81 (67), 67 (82), 55 (80), 41 (100). Exact mass calculated for $C_{16}H_{29}O_2$ (MH+): 253.2162; measured 253.2168. $[\alpha]_{25}^{D}$=+29.5º (C=1.54, CH_2Cl_2).

(4S,7Z)-Hexadec-9-en-4-olide (**8**) The (*S*)-enantiomer was made in analogous fashion from (5*R*)-dihydro-5-(hydroxymethyl)-2(3H)-furanone (Sigma-Aldrich, St. Louis, MO, USA). Exact mass calcd. for $C_{16}H_{29}O_2$ (MH+): 253.2162; measured 253.2166. Ee 98.5%, retention time on ß-Dex 225 column, 40.52 min. $[\alpha]_{25}^{D}$=−27.3º (C=1.51, CH_2Cl_2).

Field Bioassays of Synthetic Compounds We tested responses of adult *D. c. californicus* to the enantiomers and the racemate of (*Z*)-9-hexadec-9-en-4-olide in field bioassays, using funnel traps composed of a 1.9 l plastic funnel attached to a clear plastic jar (1.9 l, General Bottle Supply Company, Los Angeles, CA, USA). The funnel spout was removed, leaving a 3.5-cm-diam hole at the bottom. A ~10 cm hole was cut into the threaded lid of the jar, and the funnel spout was hot-melt glued to the lid so that the spout emptied into the jar. Traps were suspended from *S. nigra* ssp. *canadensis* branches, and traps were not in contact with other branches or leaves. Pheromone lures consisted of clear low-density polyethylene press-seal bags ("Zipper" seal sample bags, 2″×3″, 0.002″ wall thickness, #01-816- 1A; Fisher Scientific, Pittsburgh, PA, USA), which were loaded with solutions of pheromone or solvent only (control), sealed, and suspended with wire from the side of the funnel.

We monitored flight activity of *D. c. californicus* with pheromone-baited sentinel traps at two sites in the vicinity of the site where we collected pupae in 2010 (Table 1: sites 1 and 2). Sentinel traps were baited with 10 mg of either (4*R*,9*Z*)- or (4*S*,9*Z*)-hexadec-9-en-4-olide [(*R*)- and (*S*)- desmolactone, respectively] in 490 µl of absolute ethanol (500 µl total volume). The sentinel traps were set on 20 March 2011, within the seasonal activity period of the species (Linsley and Chemsak, 1972). Nine adult male *D. c. californicus* were captured in sentinel traps within 7 d. Full field bioassays were set on 27 March, and ran until 24 April 2011 (variable skies, light precipitation, air temperatures 4–34°C, maximum wind speed 40 km/h). Each replicate comprised 4 traps, respectively baited with 10 mg of (*R*)-desmolactone in 490 µl ethanol, 10 mg of (*S*)-desmolactone in 490 µl ethanol, 20 mg of a racemic blend of (*R*)- and (*S*)- enantiomers in 480 µl of ethanol, or 500 µl ethanol as a control. Previous work indicated that *D. c. californicus* individuals were not attracted to ethanol in the quantities released by these lures (AMR, data not shown). On 9 April, we added replicates at two additional sites in elderberry savannas, and these field bioassays also ran until 24 April 2011 (Table 1: sites 3 and 4). In all bioassays, traps were separated by ~10 m, with pheromone and control treatments assigned randomly to traps.

Traps were checked for beetles every 7 d, at which time lures were replaced and the traps were shifted one position along the transect to control for location effects. Captured beetles were sexed using the sexually dimorphic characters of antennal length, width of the elytra, and shape of the terminal abdominal sternite (Linsley and Chemsak, 1972). Differences between trap treatments in the number of beetles captured per trap per week were tested with the nonparametric Friedman's test (blocked by site; PROC FREQ with CMH option; SAS Institute, 2001) because assumptions of analysis of variance were violated by heteroscedasticity (Sokal and Rohlf, 1995). Differences between pairs of means were tested with the REGWQ means-separation test to control maximum experimentwise error rates (SAS Institute, 2001).

Voucher specimens of *D. c. californicus* have been deposited at the Entomology Research Museum at the University of California, Riverside (voucher # UCRC ENT 293596–293625).

Results

Identification of the Pheromone Analysis of aeration extracts collected from adult beetles held on elderberry stems revealed a small sex-specific peak in extracts from females that elicited a strong response from antennae of male beetles in GC-EAD analyses (Fig. 2), with a retention

Table 1 Location of field bioassays (City of Redlands, San Bernardino Co., CA, USA). GPS coordinates indicate the position of the first trap in each replicate

Site	Nearest road	Latitude	Longitude	Elevation
1	E. San Bernardino Ave.	34.076640	−117.198271	390 m
2	E. San Bernardino Ave.	34.075510	−117.142481	480 m
3	Nevada St.	34.070682	−117.220322	360 m
4	Texas St.	34.087708	−117.190968	395 m

index of 2090 on a relatively nonpolar DB-5 column and 2827 on a polar DB-Wax column. The compound showed a significant molecular ion (10% of base peak) at *m/z* 252 (Fig. 3), for possible molecular formulae of $C_{17}H_{32}O$ or $C_{16}H_{28}O_2$. Treatment with Pd/C and hydrogen cleanly reduced the compound to a new peak with a small molecular ion at *m/z* 254, indicating the presence of one C=C double bond (Fig. 4). The spectrum of the reduced compound was dominated by the base peak at *m/z* 85, suggestive of a γ-lactone with a C_{12} alkyl substituent on the γ-carbon. Because there was a significant *m/z* 85 ion (~50%) in the mass spectrum of the natural compound, it seemed likely that the C=C double bond was in the side chain rather than in the lactone ring. Furthermore, the mass spectrum of the natural compound bore strong similarities to the spectrum of a homolog, (*Z*)-9-octadecen-4-olide, a pheromone component of the currant stem girdler *Janus integer* (Cossé et al., 2001). The position of the double bond was determined by dimethyldisulfide (DMDS) derivatization of the double bond (Fig. 5). The mass spectrum of the resulting adduct gave a molecular ion at *m/z* 346 (16%) as expected, and diagnostic fragments at *m/z* 201 (21%) and 145 (base peak), from cleavage of the DMDS adduct of the isomer with a double bond between carbons 5 and 6 of the 12-carbon side chain. The stereochemistry of the double bond was confirmed by synthesis of an authentic standard of (*Z*)-9-hexadecen-4-olide, followed by scrambling of the double bond by treatment with thiophenol (Attygalle, 1998). The resulting solution, containing a mixture of the (*E*)-and (*Z*)-isomers of desmolactone, exhibited only a single peak on DB-5 MS or DB-17 GC columns. However, the epoxides of the (*E*)- and (*Z*)-isomers, generated by treatment of the mixture of isomers with *m*-chloroperbenzoic acid, were separated to baseline. Specifically, the mixture consisted of a 69:31 mixture of the earlier eluting (*E*)-diastereomers as one peak (retention time 21.75 min), and the later-eluting (*Z*)-diastereomers as two overlapped peaks (retention times 21.88 and 21.90 min). The later eluting diastereomers were confirmed to be the (*Z*)-isomers by analysis of an epoxidized sample of synthetic desmolactone. Epoxidation and analysis of a headspace extract collected from female beetles gave two overlapped peaks at 21.88 and 21.90 min, with no sign of a peak at 21.75 min, thus confirming that the double bond in the insect-produced compound had exclusively the (*Z*)-configuration. Finally, the absolute configuration of the insect-produced compound was determined to be (*R*) by analysis on a chiral stationary phase ß-Dex 225 GC column (Fig. 6). On this column, the enantiomers were resolved to baseline, whereas all attempts to resolve them on a Cyclodex B® column were unsuccessful.

Antennae of males also responded to (*S*)-desmolactone, although the response was smaller than that to the insect-produced enantiomer (data not shown). The antennae of females did not respond to any insect-specific components

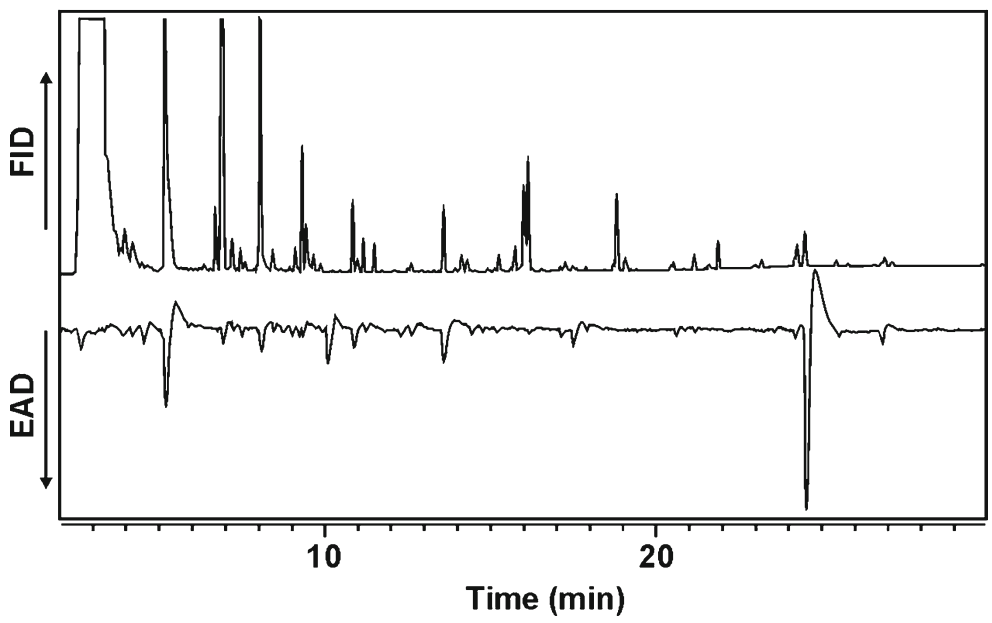

Fig. 2 Coupled gas chromatography-electroantennogram analysis of an extract of headspace odors from a female *Desmocerus c. californicus*. Upper trace is the chromatogram, lower inverted trace is the electroantennogram signal from the antenna of a male beetle. DB-5 column, 40ºC/1 min, then 10ºC/min to 275ºC, hold 40 min

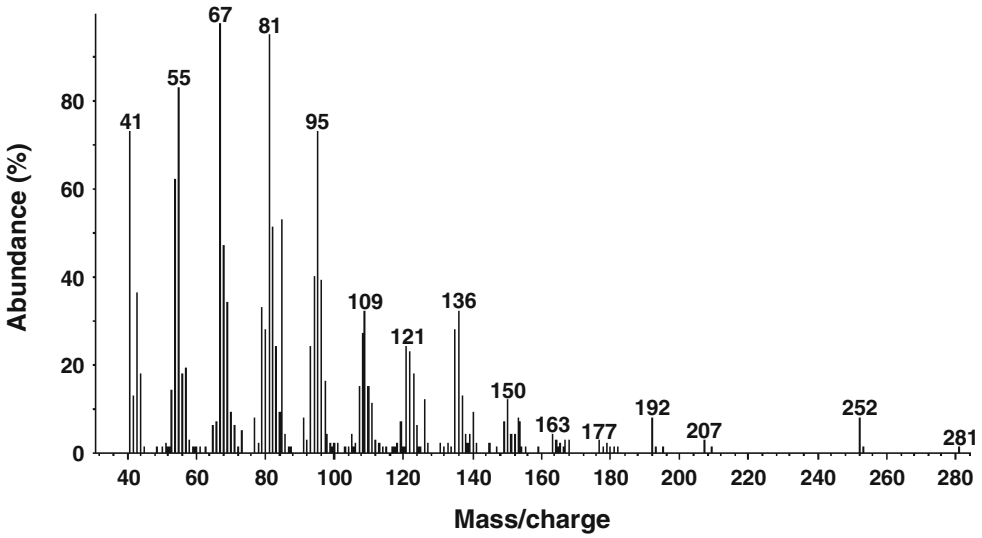

Fig. 3 EI mass spectrum (70 eV) of the insect-produced compound that elicited a strong electroantennogram response in Fig. 2

in the extracts of headspace volatiles from either sex. The extracts of headspace volatiles of male beetles did not contain (*R*)- or (*S*)-desmolactone or any other insect-specific compounds. However, several other compounds in the aeration extracts elicited small responses from the antennae of both sexes. These compounds, which included (*E*)-2-hexenal, benzaldehyde, benzyl alcohol, and (*E*)-4,8-dimethyl-1,3,7-nonatriene, also were present in control extracts prepared from aerations of elderberry stems without beetles, indicating that they were from the host plant rather than the insects.

Synthesis of Desmolactone Enantiomers (Fig. 1) The alkyl chain portion of the molecule was prepared by selective alkylation of 1-bromo-3-chlorobutane by the lithium salt of 1-octyne in refluxing THF (Armstrong-Chong et al., 2004), followed by reduction of chloroalkyne **2** with P2-nickel and hydrogen. The resulting (*Z*)-1-chloroundec-4-ene **3** was converted to the corresponding Grignard reagent **4** by reaction with Mg chips in ether.

The other required intermediate was generated by conversion of (*S*)-dihydro-5-(hydroxymethyl)-2(3H)-furanone **5** to the corresponding triflate **6** by treatment with trifluoromethanesulphonic anhydride and pyridine in CH_2Cl_2. It was essential to use the triflate rather than the analogous iodide or bromide, because of the known difficulties in displacement of leaving groups in compounds with an oxygen functionality on the carbon ß to the leaving group (Lipshutz, 1994). In fact, the coupling reaction failed when it was attempted with the corresponding iodide. In contrast, reaction of the freshly prepared triflate with (*Z*)-undec-4-enylmagnesium chloride **4** in ether at −40 to −20°C, with Li_2CuCl_4 catalyst, gave the desired (4*R*,7*Z*)-hexadec-9-en-4-olide in 93% yield, with an ee >95%. The (*S*)-enantiomer was made in analogous yield and enantiomeric purity from (*R*)-dihydro-5-(hydroxymethyl)- 2(3H)-furanone.

Field Bioassays of Synthetic Compounds Traps baited with (*R*)-desmolactone captured 170 male *D. c. californicus*,

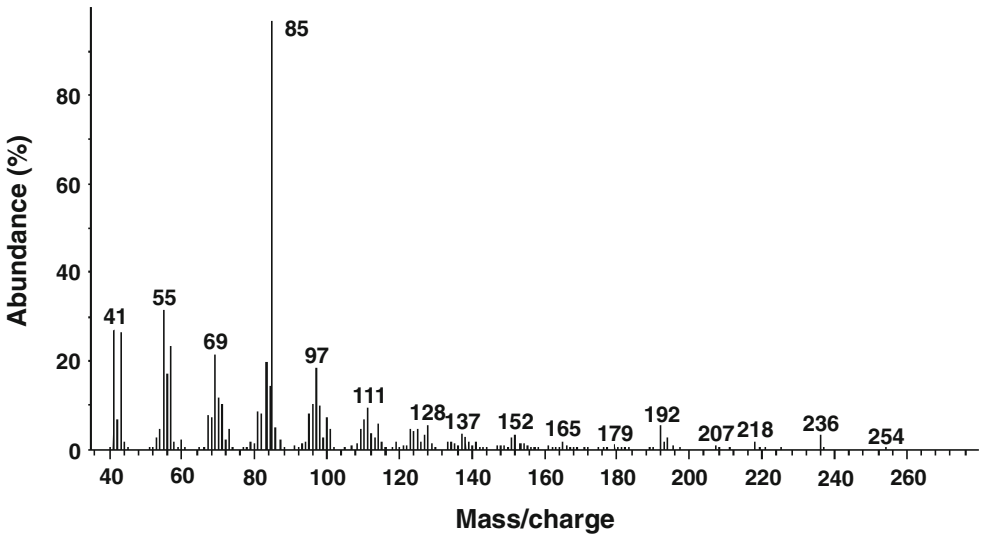

Fig. 4 EI mass spectrum (70 eV) of the insect-produced compound after catalytic hydrogenation

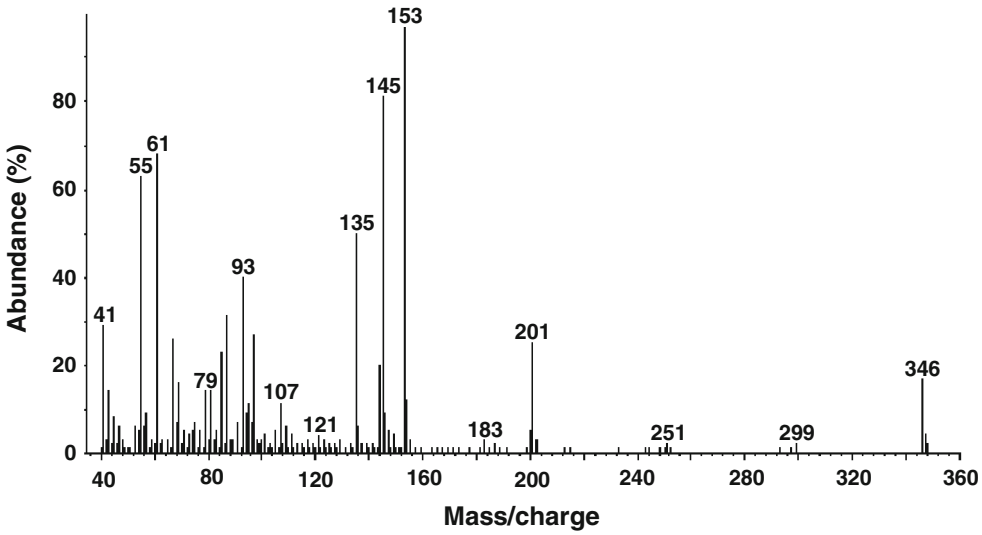

Fig. 5 EI mass spectrum (70 eV) of the dimethyldisulfide adduct of the insect-produced compound

whereas traps baited with racemic desmolactone captured 73 males at field sites in Redlands, CA, USA (Fig. 7; treatment means significantly different, Friedman's Q=34.4, $d.f.$=3, N=48, P<0.001). No beetles were captured in traps baited with the (*S*)-enantiomer or in control traps. As many as 29 male beetles were caught per trap per week, with a mean of 10.1±1.8 (SE) beetles per trap per week (excluding treatments that captured no beetles).

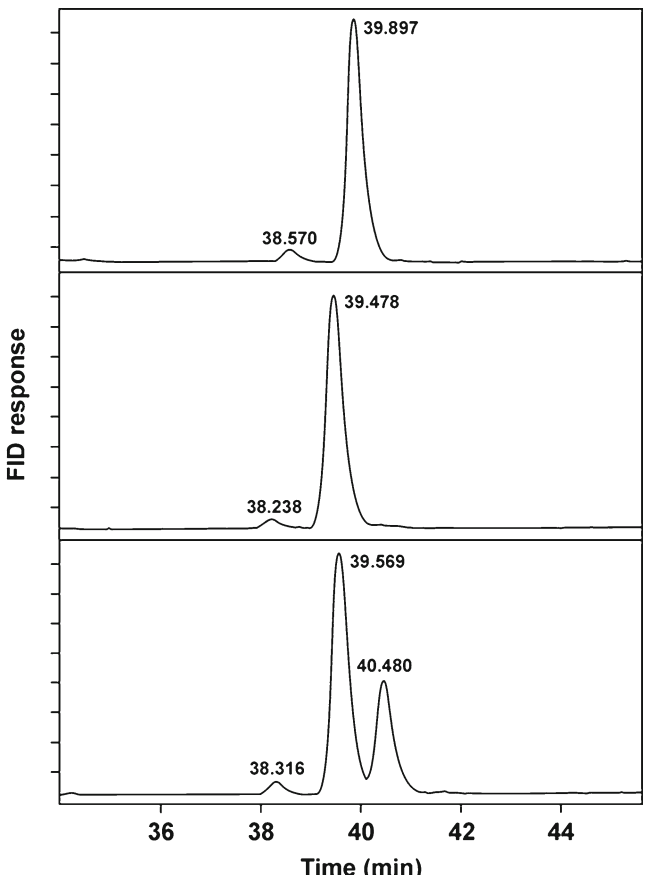

Fig. 6 GC analyses of insect-produced and synthetic desmolactone enantiomers on a chiral stationary phase β-Dex 225 column (185ºC isothermal). Coinjection with standards revealed that the insect-produced compound has the (*R*) configuration. *Top*: insect-produced compound. *Middle*: insect extract coinjected with synthetic (*R*)-desmolactone, showing only a single peak. *Bottom*, insect extract coinjected with synthetic (*S*)-desmolactone, showing two peaks

Discussion

Several asymmetric syntheses of (*Z*)-9-octadecen-4-olide, the two-carbon homolog of the *D. c. californicus* pheromone, have been reported, with the chiral center being created by regioselective ring opening of a chiral terminal epoxide (James et al., 2003; Habel and Boland, 2008) or asymmetric addition of an alkyne precursor to an aldehyde (Lin et al., 2009). However, because both enantiomers of a logical precursor, dihydro-5-(hydroxymethyl)-2(3H)-furanone, were commercially available we decided to attempt a short synthesis based on these synthons. This strategy proved successful, producing gram quantities of both enantiomers efficiently and in high stereochemical purity.

In field bioassays, traps baited with 10 mg doses of synthetic (*R*)-desmolactone captured only male *D. c. californicus*, indicating that (*R*)-desmolactone is a sex pheromone for this species. Traps baited with 10 mg doses of the (*S*)-enantiomer captured no beetles, and traps baited with the racemate captured significantly fewer beetles than traps baited the (*R*)-enantiomer alone, suggesting some degree of antagonism by the unnatural enantiomer. To date, we have not tested different doses of pheromone in field bioassays, nor is it clear how much pheromone is released by females under natural conditions. Analyses of aeration extracts suggested that the pheromone may be produced in relatively small quantities (Fig. 2), but this may be an

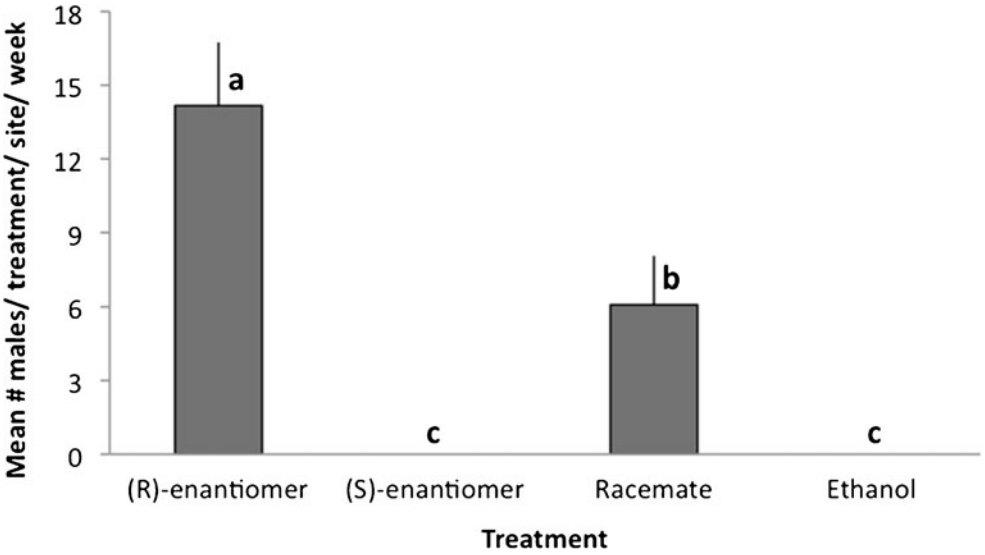

Fig. 7 Mean (±SE) number of male *Desmocerus c. californicus* captured from 27 March–24 April 2011 in elderberry savanna in Redlands, CA with funnel traps baited with 10 mg of (*R*)-desmolactone in 490 µl absolute ethanol, 10 mg (*S*)-desmolactone in 490 µl absolute ethanol, 20 mg of a racemic blend of the two enantiomers in 480 µl absolute ethanol, or 500 µl absolute ethanol (solvent control). Bars with different letters are significantly different (REGWQ test)

artifact of collecting volatile emissions under the unnatural conditions of laboratory aerations.

The fact that only males were attracted to the pheromone is analogous to the exclusive attraction of males to the female-produced pheromone of *Ortholeptura valida*, the only other lepturine species for which volatile pheromones have been identified (Ray et al., 2011). Female-produced sex pheromones also have been identified in several species of the cerambycid subfamily Prioninae (Barbour et al., 2011; Paschen et al., unpublished data; Ray et al., unpublished data). In contrast, male-produced pheromones have been identified from many species within the cerambycid subfamilies Cerambycinae, Lamiinae, and Spondylidinae, and to date, no volatile, female-produced pheromones have been identified from any species within these groups (e.g., Silk et al., 2007; Millar et al., 2009; Ray et al., 2009; Fonseca et al., 2010; Pajares et al., 2010; Mitchell et al., 2011; Teale et al., 2011). However, these observations may reflect how few pheromones are known for cerambycid species, rather than actual trends within subfamilies.

The pheromone of *D. c. californicus* represents a new structural class of cerambycid pheromone. (*R*)-desmolactone is structurally dissimilar to *cis*-vaccenyl acetate, the pheromone of the lepturine *O. valida*, although both pheromones likely are derived from fatty acid biosynthetic pathways (James et al., 2003). (*R*)-Desmolactone is a shorter chain homolog of (4*R*,9*Z*)-octadec-9-en-4-olide, the pheromone of an unrelated species, the currant stem girdler, *Janus integer* (Norton) (Hymenoptera: Cephidae) (Cossé et al., 2001), and is similar in structure to (*R*)-(*Z*)-7,15-hexadecadien-4-olide, the sex pheromone of the yellowish elongate chafer, *Heptophylla picea* Motschulsky (Coleoptera: Scarabaeidae) (Leal et al., 1996), and to (*E*)-11-tetradecen-4-olide, a male-produced sex pheromone component of two species in the braconid wasp genus *Spathius* (Hymenoptera: Braconidae) (Cossé et al., 2011).

To date, most cerambycid species for which volatile pheromones have been identified can be assigned to one of two categories based on biology and the role of volatile pheromones in mate location: either larvae develop in stressed, moribund, or recently dead hosts and volatile pheromones likely serve to expedite colonization of the ephemeral host resource (Hanks, 1999; see also Ray et al., 2011), or adult females do not readily fly, and volatile pheromones are likely used to attract mobile males to the relatively immobile females (Hanks, 1999; Barbour et al., 2011; but see Fonseca et al., 2010). *Desmocerus c. californicus* is unusual among pheromone-producing cerambycid species because it cannot be assigned to either category. Larvae develop within seemingly healthy hosts (mature *S. nigra* ssp. *canadensis*), and adults of both sexes are highly mobile and readily fly (Linsley and Chemsak, 1972; AMR, personal observation). As such, the adaptive significance of volatile pheromone production is unclear for *D. c. californicus*.

Research has shown that the pheromones of a subset of cerambycid species are strongly synergized by host plant volatiles (Silk et al., 2007; Teale et al., 2011). To date, we have not tested whether host plant volatiles may also synergize attraction to desmolactone. However, strong synergism seems unlikely in this case, for two reasons. First, desmolactone is clearly a sex pheromone, being produced by females and attracting only males, whereas all known examples of cerambycid pheromones that are synergized by host volatiles are aggregation pheromones. Second, the fact that we caught so many *D. c. californicus* in traps baited only with pheromone suggests that host plant volatiles do not form a major part of the natural attractant. In particular, this species is rarely collected, and the number of beetles caught in our bioassays was greater

than the total number of specimens in all of California's entomological museum collections (IPS, personal observation).

It is also noteworthy that unlike pheromones identified from most other cerambycid species, the pheromone of *D. c. californicus* does not appear to be a "generic" attractant that is used as a pheromone component by a number of related species (for information about generic pheromones, see Millar et al., 2009; Barbour et al., 2011; Mitchell et al., 2011). No other cerambycid species were caught in traps baited with (*R*)- or (*S*)-desmolactone in the bioassays described here. Furthermore, we did not capture any individuals of the congeners *D. palliatus* (Forster) or *D. aureipennis* Chevrolat in similar field surveys in areas of California, Missouri, and Ohio where these other species are known to occur, thus suggesting that (*R*)-desmolactone may be a species specific pheromone.

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Reduced Mating Success of Female Tortricid Moths Following Intense Pheromone Auto-Exposure Varies with Sophistication of Mating System

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Abstract Mating disruption is a valuable tool for the management of pest lepidopteran species in many agricultural crops. Many studies have addressed the effect of female pheromone on the ability of males to find calling females but, so far, fewer have addressed the effect of pheromone on the mating behavior of females. We hypothesized that mating of female moth species may be adversely affected following sex pheromone auto-exposure, due to abnormal behavioral activity and/or antennal sensitivity. Our results indicate that, for *Grapholita molesta* and *Pandemis pyrusana* females, copulation, but not calling, was reduced following pre-exposure to sex pheromone. In contrast, for *Cydia pomonella* and *Choristoneura rosaceana*, sex pheromone pre-exposure did not affect either calling or copulation propensity. Adaptation of female moth antennae to their own sex pheromone, following sex pheromone auto-exposure, as measured by electroantennograms, occurred in a species for which identical exposure reduced mating success (*G. molesta*) and in a species for which such exposure did not affect mating success (*C. rosaceana*). These results suggest that pre-exposure of female moths of certain species to sex pheromone may further contribute to the success of pheromone-based mating disruption. Therefore, we conclude that, in some species, mating disruption may include a secondary mechanism that affects the mating behavior of female moths, in addition to that of males.

Keywords Mating disruption · Tortricidae · Autodetection · Cross-adaptation · Biorational pest management

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Introduction

Mating disruption is an effective technique for the control of pest insects in a variety of settings. Successful mating disruption techniques have been established for various lepidopteran pests, including *Cydia pomonella* (Pfeiffer et al., 1993), *Grapholita molesta* (Vickers et al., 1985), and various leafroller species (Pfeiffer et al., 1993). Despite extensive research and development, the mechanisms of mating disruption need to be elucidated further. Superficially, mating disruption interferes with a male's ability to locate a female via her pheromone plume; however, the specific mode of action may be due to a number of mechanisms including, habituation to odorant, arrestment of males at high concentration of pheromone, shifting the rhythm of mating behavior, camouflage of the female plume, and competition for males between the mating disruption point source and the calling female (Cardé and Minks, 1995; Miller et al., 2006; Witzgall et al., 2008). It is important to understand the underlying mechanisms of this technology in order to maximize its efficiency and ensure continued success.

Female sex pheromone autodetection, as measured by responses of olfactory receptor neurons, is known to occur in *Panaxia quadripunctaria* (Schneider et al., 1998) and *C. pomonella* (Ansebo et al., 2005). Similarly, Stelinski et al. (2006a) and Gökçe et al. (2007) demonstrated that female *G. molesta* and *Choristoneura rosaceana* are capable of autodetection. Furthermore, intense exposure to sex pheromone had varying effects on female calling behavior in both species (Stelinski et al., 2006a; Gökçe et al., 2007). Although these investigations did not focus on female mating behavior following sex pheromone pre-exposure, it was noticed that the mating behavior of *G. molesta* and *C. rosaceana* females were affected differentially, following otherwise similar pheromone pre-exposure regimes. Specifically, the mating success of *G.*

molesta, but not that of *C. rosaceana*, appeared reduced. Based on these observations, we hypothesized that intense exposure of female moths to sex pheromone may differentially affect subsequent mating behavior, depending on whether or not the mating ritual includes hair pencil exposure during courtship (Baker and Cardé, 1979a) and the need to recognize a male aphrodisiac pheromone for mating success.

Mating has been described for several tortricid pest species. In general, males orient and fly upwind, in a zigzag pattern, toward females releasing pheromone (Marsh et al., 1978). Pulses in pheromone release, resulting in fluctuating concentration packets of pheromone, are necessary for male moths to respond (Baker et al., 1985). Once a male moth reaches a female, several behaviors may ensue in a short period of time. These can include, wing fanning, head or body contact, release of chemical cues (aphrodisiac pheromones) from specialized structures on male moths (hair-pencils), female approach, and copulation (Baker and Cardé, 1979a; Castrovillo and Cardé, 1980). *Grapholita molesta* is a well characterized tortricid pest species with a highly evolved mating ritual. Baker and Cardé (1979b) described the behavioral sequence and associated probabilities for successful mating, including the requirement of a male aphrodisiac to stimulate female acceptance. Although similar male aphrodisiac signals have not been identified in the tortricid, *Pandemis pyrusana* Kearfott, females require a male signal for mate acceptance that suggests the use of a male aphrodisiac (Curkovic et al., 2006). In contrast, both *C. rosaceana* and *C. pomonella* appear to have relatively simple mating behaviors with no evidence of a male aphrodisiac (Castrovillo and Cardé, 1980; Curkovic et al., 2006).

Despite widespread research documenting how pheromone exposures affect male moth behavior, few studies have addressed how behaviors of females are affected by sex pheromone exposure, and how this might influence the efficacy of mating disruption. In this research, we individually exposed both females and males of four tortricid moth species to their respective female sex attractants, and subsequently assessed ability of females to call and their propensity to copulate.

Methods and Materials

Insects *Cydia pomonella, Grapholita molesta,* and *Choristoneura rosaceana* were obtained from four-, five-, and 8 year-old laboratory colonies, respectively, originally collected as larvae from apple orchards in Southwest Michigan, U.S.A. *Pandemis pyrusana* were from a colony established in 1990 from a commercial apple orchard in Yakima, WA, USA (Curkovic et al., 2006); *Pandemis pyrusana*, collected from an untreated apple orchard in Wenatchee, WA, USA, were added to this colony in 2000 and 2003. All species were reared at 24°C and 60% RH on pinto bean-based diet (Shorey and Hale, 1965), under a 16:8 (L:D) photoperiod. Pupae, sorted by species and sex, emerged in 1 L plastic cages, containing a 5% sucrose solution in a plastic cup with a cotton dental wick protruding from the lid.

Chemicals and Release Devices Moths of each species were pre-exposed to emissions from dispensers containing synthetic components of the specific pheromone. Two types of pheromone dispensers were used to expose moths of each species: 1) red rubber septa (The West Company, Lionville, PA, USA), loaded with partial pheromone blends at dosages known to elicit substantial captures of male moths when deployed in sticky traps in the field; and 2) Isomate polyethylene reservoir dispensers, specifically formulated for mating disruption of each species. The septa were considered a low-release pheromone pre-exposure treatment, while the reservoir dispensers were considered a high release pre-exposure treatment. For *C. pomonella*, septa were loaded with 0.1 mg of (*E*,*E*)-8,10-dodecadien-1-ol (Stelinski et al., 2006a; 99% isomeric and chemical purity; Bedoukian Co., Danbury, CT, USA). The high release dispenser for *C. pomonella* was Isomate-C Plus, containing 205 mg of 53.0% (*E*,*E*)-8,10-dodecadien-1-ol, 29.7% dodecanol, 6.0% tetradecanol, and 11.3% inert ingredients (Shin Etsu, Tokyo, Japan). The release rate of an Isomate-C Plus dispenser is 0.02 mg of pheromone/hr (Stelinski et al., 2009). For *G. molesta*, septa were loaded with 3 μg of (*Z*)-8-dodecenyl acetate, (*E*)-8-dodecenyl acetate, and (*Z*)-8-dodecen-1-ol in a 100: 6 :10 blend (Baker and Cardé, 1979b; pheromone components >95% purity; Shin Etsu). For *G. molesta*, Isomate-M Rosso dispensers, containing 250 mg of 88.5% (*Z*)-8-dodecen-1-yl-acetate, 5.7% (*E*)-8-dodecen-1-yl-acetate, 1.0% (*Z*)-8-dodecen-1-ol, and 4.8% inert ingredients, were used (Shin Etsu). The release rate of an Isomate-M Rosso dispenser is 0.05 mg of pheromone/h (Stelinski et al., 2009). For *C. rosaceana*, rubber septa were loaded with 0.485 mg of (*Z*)- and 0.015 mg (*E*)-11-tetradecenyl acetates (92.2: 3.0 ratio of *Z*: *E*), and 0.026 mg of (*Z*)-11-tetradecenol (Hill and Roelofs, 1979; pheromone components >95% isomeric and chemical purity; Shin Etsu). The high release exposure dispenser for *C. rosaceana* was Isomate OBLR/PLR Plus, containing 274 mg of 93.4% (*Z*)-11-tetradecenyl acetate, 5.1% (*E*)-11-tetradecenyl acetate, and 1.5% (*Z*)-9-tetradecenyl acetate (Shin Etsu). The approximate release rate of this dispenser is 0.04 mg of pheromone/h (Stelinski et al., 2009). For *P. pyrusana*, red septa were loaded with 0.4 mg of a 94: 6 blend of (*Z*)-11-tetradecenyl acetate: (*Z*)-9-tetradecenyl acetate (Roelofs et al., 1977; >95% Purity, Shin Etsu). For this species, the high dosage dispenser was also Isomate OBLR/PLR, as it releases the same major pheromone component for both *P. pyrusana* and *C. rosaceana* and is intended for simultaneous

mating disruption of both species. Pheromone blend solutions used to load rubber septa were prepared in HPLC grade hexane and stored at −18°C between exposure treatments. All Isomate dispensers were aged in a fume hood at 24°C for 3 week prior to pre-exposure experiments, to allow for flash evaporation of pheromone that may have accumulated on the surface during packaging and storage.

Pheromone Pre-exposure Protocol Moths destined for pre-exposure were placed in 1-l plastic assay chambers, equipped with two 0.64 cm openings in their lids [See Fig. 1 in Stelinski et al. (2006a)]. Glass inlets and outlets were affixed to the lids, allowing for air to pass through the chambers. Carbon-filtered (filter model 100 Safe Glass Hydrocarbon Trap, Chromatography Research Supplies, Louisville, KY, USA; 50 ml/min) air

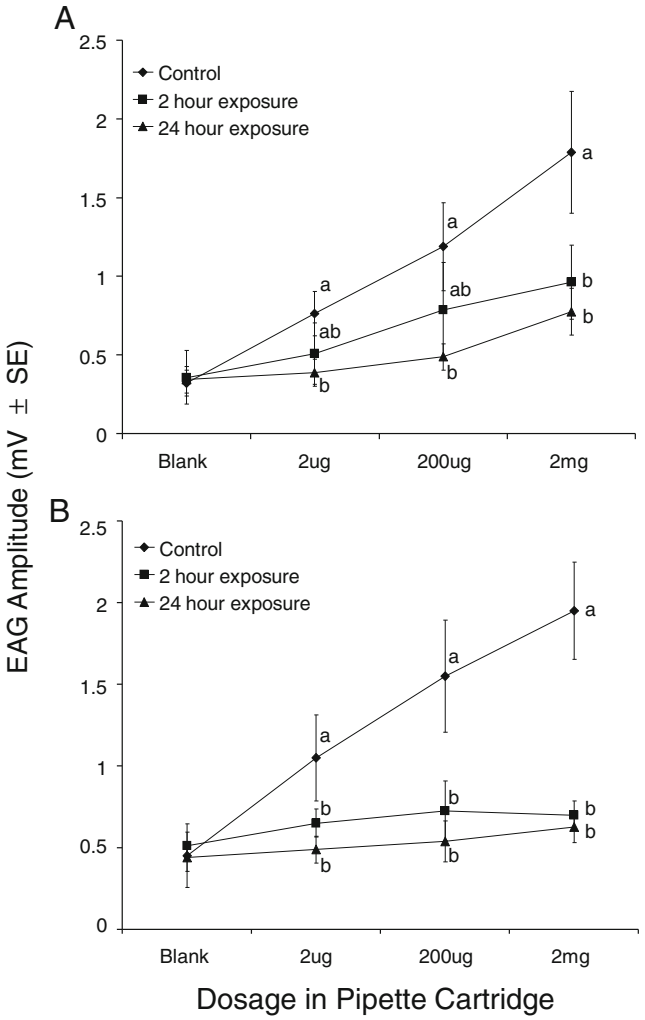

Fig. 1 Electroantennogram (EAG) responses of female *Grapholita molesta* (**a**) and *Choristoneura rosaceana* (**b**) to different dosages of synthetic sex pheromone, following non-exposure (control) or pre-exposure to sex pheromone for 2 or 24 hr. Differences between EAG responses, for a given dosage, are indicated by different letters. *G. molesta* were exposed to Isomate-M Rosso dispensers and *C. rosaceana* were exposed to Isomate OBLR/PLR Plus dispensers

was initially passed through Teflon chambers (described in Stelinski et al., 2003a) containing a given pheromone pre-exposure treatment, described above. From these pheromone-source chambers, air flowed into the exposure chambers, containing moths, via 20 cm of Teflon tubing (Stelinski et al., 2006b). Exposure chambers contained exit valves for air; thus, there was a constant flow of air through the exposure chambers during the pre-exposure period, creating a constant pheromone exposure concentration per unit of air, as described in Stelinski et al. (2003a). Teflon chambers, free of pheromone sources and connected to moth pre-exposure chambers, served as a negative control for each species and dispenser treatment. Each species was tested in separate experiments. Exposure chambers were housed in a wind tunnel, similar to that described in Stelinski et al. (2004), which evacuated pheromone-laden air continuously at 0.5 $m.sec^{-1}$ during pre-exposures. The tunnel was housed in a temperature- and photoperiod-controlled (16:8, L:D) room, maintained at 23°C and 50-70% RH, with light intensity ranging between 3 and 10 lux.

For each species, experiments were conducted either by exposing females only and pairing them with unexposed males for subsequent behavioral assays, or *vice versa*. When one sex was exposed, the other sex was obtained from the rearing method described above without previous exposure to synthetic pheromone. Durations of either two or 24 h exposure were conducted for each pheromone pre-exposure treatment and for each species. For the 2 h pre-exposure treatment, moths in chambers were exposed to various treatments (no pheromone control, low dosage dispenser, high dosage dispenser) for 2 h prior to the period of maximal calling behavior of females and sexual responsiveness by males. For *C. pomonella*, *C. rosaceana*, and *P. pyrusana*, exposures were initiated 2–2.5 h prior to the end of the 16 h photophase. For *G. molesta*, exposure was initiated 5 h prior to the end of the 16 h photophase. For 24 h exposures, durations were adjusted, such that moths were similarly pre-exposed starting 24 h prior to onset of maximal diel sexual responsiveness, as described above. In assays in which females were pre-exposed, and males left unexposed, 50 replicates of females and males were bioassayed. In cases in which males were exposed, and females were not, 20 replicates of pairs were bioassayed. The male exposure treatment served as a type of positive control, to confirm that pheromone exposures affected moth behavior, as it is well established that such exposures decrease pheromone-mediated behavior of male moths (Witzgall et al., 2008).

Behavioral Assays Following Pheromone Exposure Directly following pre-exposure, pairs of moths (one sex pheromone exposed and the other not) were placed into plastic cylindrical cages, similar to those described in Curkovic et al. (2006). In brief, cages were transparent, 30 cm length x

10 cm diam. tubes of acetate sheet, sealed at both ends with fine wire mesh, which prevented moth escape but allowed airflow. These cages were placed into the wind tunnel under conditions described above, with airspeed of 0.3 m.sec^{-1}. Cages were suspended approximately 30 cm above the tunnel floor, using ring stands in an orientation that allowed direct airflow through the cylinders. Mating pairs were established initially by placing females at the upwind end of the tubes and males at the down-wind end, and the ends sealed with wire-mesh caps. Mating pairs of moths within cylinders were observed over a period of 60 min, during which incidences of female calling or female–male copulation were recorded. Five to eight tubes were observed simultaneously during a given session. Calling behavior of female moths was noted by the characteristic posture of females, of wings elevated slightly and displaced laterally, with the abdomen concurrently raised, as described (Baker and Cardé, 1979a; Curkovic et al., 2006). Copulation was recorded when a male clasped a female's abdomen. In experiments with pheromone-exposed females and un-exposed males, males behaved normally by walking toward calling females, wing fanning, and attempting copulation.

Antennal Response of Female Moths Following Pheromone Pre-exposure We sought to determine whether auto-exposure of female moths to sex pheromone resulted in antennal adaptation in the subsequent response to sex pheromone. Two (of the four) representative species were tested. Female moths of the two species were pre-exposed to the high rate of pheromone, for either two or 24 h, as described above. Females in blank untreated chambers, for the same times as the exposure treatments, served as controls. Each species was exposed to the respective mating disruption dispenser, described above. Soon after exposure (1–2 min), antennal responses were tested by electroantennogram (EAG). The EAG system (Syntech, Kirchzarten, Germany) and test protocols have been described elsewhere (Stelinski et al., 2003a, b). EAG cartridges were made by pipetting various dosages (2 μg–2 mg) of pheromone in hexane (20 μl total solution) onto 1.4×0.5 cm strips of Whatman No. 1 filter paper. The blends used were the same as in the low dosage pre-exposure lures, described above. After 5 min. in a fume hood for solvent evaporation, pheromone-treated filter paper strips were inserted into disposable glass Pasteur pipettes. EAGs were measured as the maximum amplitude of depolarization to 1-ml puffs of air through EAG-cartridges directed over live-insect preparations.

Female moths of each species were 2–4 d-old when tested. A live insect was restrained on a 3.5 cm diam. Petri dish, filled with wax, by placing a strip of clay (8×3 mm) over the thorax and abdomen. The terminal two segments of the antenna used for recording EAGs were excised, and the recording electrode attached at the severed end. The reference electrode was gently inserted into the head capsule near the base of the antenna used for recording. EAGs were measured for 8 moths per pheromone pre-exposure treatment or control. Solvent-only stimulations (using filter paper impregnated with 20 μl of hexane) were administered as a control for subsequent pheromone stimulations. Each replicate moth was stimulated with the blank solvent, followed by each successive pheromone dosage within the range of dosages administered (Fig. 1a,b). Successive stimulations were applied 12–20 sec apart, to minimize potential onset of additional adaptation due to the stimulus puff.

Data Analysis To investigate the effects of pheromone dosage and exposure interval on the probability of calling and copulation by four moth species, logistic regression was used and significance tested using the log-likelihood ratio χ^2 (The LOGISTIC Procedure, SAS 9.1). Calling or copulation data were analyzed separately as dependent variables, with dosage, exposure duration, and the interaction between dosage and exposure duration, considered the independent variables (SAS INSTITUTE, 2005). EAG data were subjected to analysis of variance (ANOVA), and differences in pairs of means over time, and between treatments, were separated using Tukey's multiple comparison tests (SAS INSTITUTE, 2005). Since EAG responses of female moths exposed to clean air for two or 24 h were not different, these were combined as a single control for the pheromone exposure treatments for each species.

Results

Effect of Pheromone Pre-exposure on Male Moths To confirm that pheromone exposure dosages were sufficient to affect male moth behavior, we conducted calling and copulation assays with pre-exposed males (Table 1). In all cases, calling behavior of females was unaffected. Copulation, for each species, was greatly reduced following 2 h exposure to the lowest dosage of pheromone (Table 1). Copulation frequency was zero or near zero for each species after 24 h pheromone pre- exposure (Table 1).

Effect of Pheromone Pre-exposure on Female Moths *Choristoneura rosaceana* and *Cydia pomonella* females appeared unaffected in calling or copulation behavior (or interaction between the two), at either dose or duration of pheromone pre-exposure (Tables 2 and 3). The average proportion of calling among all treatments for *C. rosaceana* and *C. pomonella* females was 84 and 82%, respectively, while the average proportion of copulation among all treatments was 80 and 80.6%, respectively.

Table 1 Proportions of female calling and copulation observed in four species of tortricid moths in which males were pre-exposed to female sex pheromone and then mated with naive females. Males were pre-exposed for two or 24 hr at either low or high doses of female pheromone

[a]Denotes an effect (relative to the control of untreated males; $P<0.05$, log-likelihood ratio χ^2) of pre-exposure on calling or copulation

		Proportion of females exhibiting					
	Hours of pre-exposure	Calling			Copulation		
		Control	Low Dose	High Dose	Control	Low Dose	High Dose
Grapholita molesta	2	0.70	0.65	0.80	0.65	0.20[a]	0.00[a]
	24	0.70	0.80	0.75	0.60	0.00[a]	0.00[a]
Pandemis pyrusana	2	0.60	0.70	0.55	0.50	0.35[a]	0.00[a]
	24	0.65	0.75	0.85	0.60	0.00[a]	0.00[a]
Choristoneura rosaceana	2	0.85	0.80	0.75	0.75	0.35[a]	0.00[a]
	24	0.90	0.85	0.85	0.80	0.00[a]	0.00[a]
Cydia pomonella	2	0.75	0.85	0.80	0.70	0.55[a]	0.10[a]
	24	0.90	0.80	0.75	0.80	0.00[a]	0.00[a]

Similarly, dose, exposure duration, and the interaction between these factors, had no effect on calling for *G. molesta* and *P. pyrusana* females (Tables 2 and 3). However, the proportion of females that copulated was affected for both species. The proportion of copulations for *G. molesta* was affected by dosage, exposure duration, and the interaction of these two variables (Table 3). For this species, copulation success was reduced by about 40% in the high dose treatment, following 2 h pre-exposure, and by 53 and 63% for the low and high dosages, respectively, after 24 h pre-exposure (Table 2). For *P. pyrusana*, the proportion of females that copulated was affected by exposure duration, and the interaction of exposure duration and dosage, but not by dosage (Tables 2 and 3). For *P. pyrusana*, there was no reduction in copulation following 2 h pre-exposure to either dosage, but there was a reduction following 24 h pre-exposure to either dosage (Table 2).

Antennal Response of Female Moths Following Pheromone Pre-exposure For *G. molesta* females, pheromone pre-exposure for 24 h reduced ($P<0.05$) antennal responses to all pheromone dosages tested (range: 2 μg–2 mg), compared with the control (Fig. 1a). For the 2-h pre-exposure, EAG responses were reduced ($P<0.05$) only to the two mg cartridge dosage (Fig. 1a). For *C. rosaceana* females, antennal responses to the full dosages tested were reduced ($P<0.05$), compared with the control, following either two or 24 h pre-exposure (Fig. 1b).

Discussion

Humans rely on artificial methods of pest control to maintain food production. With the threat of evolution of pesticide resistance (Bates et al., 2005), and the increasing food requirements of the human population (Pimentel et al., 1994), ecologically responsible pest management methods are imperative. Pheromone-based mating disruption, which exploits natural behaviors to control insect pests, has been a valuable tool for crop management for nearly two decades

Table 2 Proportions of calling and copulation observed among females of four tortricid species in which females were pre-exposed to female sex pheromone and then caged with naïve males. Females were exposed for two or 24 hr at either low or high doses of female pheromone

[a]Denotes an effect (relative to the control of untreated females; $P<0.05$, log-likelihood ratio χ^2) of pre-exposure on calling or copulation

		Proportion of females exhibiting					
	Hours of exposure	Calling			Copulation		
		Control	Low Dose	High Dose	Control	Low Dose	High Dose
Grapholita molesta	2	0.74	0.76	0.64	0.68	0.66	0.40[a]
	24	0.78	0.82	0.74	0.72	0.34[a]	0.26[a]
Pandemis pyrusana	2	0.64	0.68	0.64	0.56	0.62	0.62
	24	0.70	0.68	0.60	0.60	0.42[a]	0.32[a]
Choristoneura rosaceana	2	0.88	0.78	0.80	0.82	0.74	0.76
	24	0.86	0.88	0.84	0.80	0.82	0.84
Cydia pomonella	2	0.78	0.86	0.84	0.74	0.82	0.84
	24	0.86	0.80	0.78	0.80	0.78	0.86

Table 3 Statistics for the effects of dosage, duration, and the interaction of dosage and duration, on proportion calling and copulation observed in four species of tortricid moths, in which females were pre-exposed to female sex pheromone and then caged with naïve males. X^2 statistics, degrees of freedom and the P-value are shown

	Statistics							
	Calling				Copulation			
	Grapholita molesta	*Pandemis pyrusana*	*Choristoneura rosaceana*	*Cydia pomonella*	*Grapholita molesta*	*Pandemis pyrusana*	*Choristoneura rosaceana*	*Cydia pomonella*
Dosage	X^2=2.682	X^2=0.915	X^2=0.956	X^2=0.140	X^2=26.259	X^2=2.437	X^2=0.251	X^2=2.014
	df=2	df=2	df=2	df=2	df=2	df=2	df=2	df=2
	P>0.05	P>0.05	P>0.05	P>0.05	P<0.001	P>0.05	P>0.05	P>0.05
Duration	X^2=1.692	X^2=0.019	X^2=0.722	X^2=0.088	X^2=5.669	X^2=7.437	X^2=0.944	X^2=0.077
	df=1	df=1	df=1	df=1	df=1	df=1	df=1	df=1
	P>0.05	P>0.05	P>0.05	P>0.05	P=0.017	P=0.008	P>0.05	P>0.05
Dosage*	X^2=0.153	X^2=0.561	X^2=1.2383	X^2=2.186	X^2=6.240	X^2=6.157	X^2=.993	X^2=0.748
Duration	df=2	df=2	df=2	df=2	df=2	df=2	df=2	df=2
	P>0.05	P>0.05	P>0.05	P>0.05	P=0.044	P=0.046	P>0.05	P>0.05

(Witzgall et al., 2011). It is likely to become increasingly prevalent in agro-ecosystems as knowledge of pheromone chemistry increases and technology for synthesizing and releasing pheromone blends becomes more available and affordable. Understanding the mechanisms of mating disruption is vital to its development, continued success, and sustainability. The current investigation explored the possibility of a new mechanism of mating disruption that specifically affects female moths. We tested the hypothesis that intense exposure to pheromone may affect the propensity of female moths to copulate.

Early work on female Lepidoptera exposed to their own pheromone proved that females of certain species are capable of autodetection, and that this may provide some information to females. Palanaswamy and Seabrook (1978) examined the behavioral responses of female *Choristoneura fumifera* in airstreams containing pheromone and found that females autodetected pheromone and, more importantly, were stimulated to oviposit. Based on similar experiments with *Trichoplusia ni* (Birch, 1977), Palanaswamy and Seabrook (1978) showed that *C. fumifera* females oviposit a minimum number of eggs before they disperse. These results indicated that female pheromone provides information to female moths that, in turn, initiates an adaptive behavioral response. Our experiments examined the behavior of females of four species of tortricid moths following exposure to pheromone. We found that, while the incidence of calling behavior was not reduced by pre-exposure to pheromone in any of the four species tested, the proportion of females that copulated following pheromone exposure was reduced in two of the four species. That prior exposure to pheromone resulted in reduced copulation proportions in *G. molesta* and *P. pyrusana*, suggests that, in these species at least, mating disruption using pheromone may simultaneously affect both male and female moths. Our results also indicated that the more intense (higher dosage) and prolonged the exposure to pheromone, the greater the likelihood of affecting female mating behaviors in these species. The combination of dosage and duration also appears to be a significant factor in mating disruption of male codling moths (Judd et al., 2005).

It is curious that *G. molesta* females were affected by pre-exposure dosage, whereas *P. pyrusana* females were not. The lack of effect on *P. pyrusana* females may indicate that they have a higher tolerance before exposure to sex pheromone modifies their behavior. This is supported by the result that *P. pyrusana* females required 24-hr exposure, to either low or high dosages of pheromone, before their behavior changed. This may indicate that *P. pyrusana* is better adapted for mating success at outbreak population levels when female pheromone is likely to be at higher concentrations. Alternatively, this difference in effect of dose and exposure between *G. molesta* and *P. pyrusana* females may be an artifact of differences in the physical properties of the pheromone components of these two species or differences in the effects of minor components on behavioral habituation. A possible adaptive explanation for the loss of female receptivity to mating in these species is that females perceive high levels of sex pheromone as an indicator of high competition that is unsuitable for reproductive success and thus may restrict their receptiveness to copulation.

An alternative interpretation for pre-exposure to pheromone affecting the behavior of female *G. molesta* and *P. pyrusana*, is that pre-exposed females may be unable to sense their conspecific male's aphrodisiac pheromone appropriately. Our EAG experiments indicated that adaptation

of antennal responses, following sex pheromone pre-exposure, occurred both in a species (*G. molesta*) requiring a male aphrodisiac as part of the courtship, as well as in a species (*C. rosaceana*) with a simpler courtship system. Of the four moth species investigated here, a specific male aphrodisiac pheromone has been identified only for *G. molesta*. Its aphrodisiac components include (*E*)-cinnamate, mullein, methyl jasmonate, and methyl-(*Z*)-epijasmonate (Nishida et al., 1982), the structures of which are quite distinct from those of the main female pheromone components, (*Z*)-8-dodecenyl acetate, (*E*)-8-dodecenyl acetate, and (*Z*)-8-dodecen-1-ol (Roelofs et al., 1969). Therefore, any sensory cross-adaptation would need to be broad. Cross-adaptation of neurons to odor stimuli has been shown in numerous studies, indicating that processing of different odor stimuli may share common physiological pathways (Daniel et al., 1994; Takeuchi et al., 2003; Gottfried et al., 2006; Anton et al., 2011). Trona et al. (2010) recently showed that structurally different sex pheromone and plant odor compounds interact in *C. pomonella* via an across-fiber coding pattern, with perception of these distinct chemicals relayed through projection neurons from ordinary glomeruli and from the macroglomerular complex. Further research is needed to determine the exact mechanism responsible for the reduced copulation of females following exposure to pheromone.

Copulation propensity of *G. molesta* and *P. pyrusana* females was affected by exposure to sex pheromone, while copulation appeared unaffected by the same treatment in *C. pomonella* and *C. rosaceana*. We find it interesting that this pattern is congruent with the respective susceptibilities of these species to pheromone mating disruption (reviewed in Gut et al., 2004). Reduction of female copulation propensity may be an additional mechanism of mating disruption that renders certain species more susceptible than others to this tactic. However, there are potential ecological consequences that need to be considered. Mating disruption could lead to destabilizing selection for utilization and attraction to abnormal pheromone blends (Evenden and Haynes, 2001). If altered mating success following exposure to pheromone is a result of an inability of females to perceive male aphrodisiacs, then females that are inherently less influenced by male aphrodisiacs during mate acceptance may be favored over females that are more strongly influenced and hence only accept males after detection of the aphrodisiac. Therefore, it is possible that, in an environment continuously treated with synthetic pheromone, selection would deemphasize the role of male aphrodisiacs in mating, and consequently reduce the efficacy of mating disruption.

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A Silica Gel Based Method for Extracting Insect Surface Hydrocarbons

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Abstract Here, we describe a novel method for the extraction of insect cuticular hydrocarbons using silica gel, herein referred to as "silica-rubbing". This method permits the selective sampling of external hydrocarbons from insect cuticle surfaces for subsequent analysis using gas chromatography–mass spectrometry (GC-MS). The cuticular hydrocarbons are first adsorbed to silica gel particles by rubbing the cuticle of insect specimens with the materials, and then are subsequently eluted using organic solvents. We compared the cuticular hydrocarbon profiles that resulted from extractions using silica-rubbing and solvent-soaking methods in four ant and one bee species: *Linepithema humile*, *Azteca instabilis*, *Camponotus floridanus*, *Pogonomyrmex barbatus* (Hymenoptera: Formicidae), and *Euglossa dilemma* (Hymenoptera: Apidae). We also compared the hydrocarbon profiles of *Euglossa dilemma* obtained via silica-rubbing and solid phase microextraction (SPME). Comparison of hydrocarbon profiles obtained by different extraction methods indicates that silica rubbing selectively extracts the hydrocarbons that are present on the surface of the cuticular wax layer, without extracting hydrocarbons from internal glands and tissues. Due to its surface specificity, efficiency, and low cost, this new method may be useful for studying the biology of insect cuticular hydrocarbons.

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Introduction

Cuticular hydrocarbons (CHCs) form thin hydrophobic layers on the cuticular surface of most insects and other arthropods, and thus play an important role in maintaining water balance and preventing lethal desiccation (Howard and Blomquist, 2005; Blomquist and Bagnères, 2010). However, in many insects and arthropods, CHCs also function as semiochemicals that contain information that is transferred among individuals of the same or different species (Blomquist and Bagnères, 2010). The CHCs of social insects, in particular, have received a great deal of attention because of their role in modulating various types of communication associated with colony membership, hierarchical dominance, fertility status, and task group membership (Blomquist and Bagnères, 2010; Liebig, 2010). The nestmate recognition of some social insects is believed to involve the matching of a "label" (i.e., the chemical profile containing the nestmate cues) with a "template" (i.e., the neural representation of the colony odor stored in the memory) (Vander Meer and Morel, 1998; van Zweden and d'Ettorre, 2010). In such studies, elucidating the CHC profile on the insect cuticular surface has been a crucial step to understand characteristics of the chemical labels that may play important roles in nestmate recognition.

Several different techniques have been developed for the extraction and analysis of insect CHCs, particularly those based on gas chromatography (GC). The most common method of extraction of CHCs involves soaking or rinsing recently killed insects in nonpolar organic solvents, such as

pentane or hexane. The hydrocarbons dissolved in the solvent can be readily separated from polar compounds by silica gel column chromatography (Blomquist, 2010). This method, herein referred to as "solvent-soaking", allows the recovery of relatively large amounts of CHCs from individual insects, which is often necessary for subsequent chemical analyses (e.g., mass spectrometry, chemical derivatization) or bioassays. Several solvent-free extraction methods have been developed for GC analysis. For example, the so-called solid phase injection techniques utilize a pyrolysis unit or sealed glass capillary tubes to insert pieces of insect cuticle directly into the GC injector port, in which the CHCs are thermally vaporized from the insect cuticle (Brill and Bertsch, 1985; Bagnères and Morgan, 1990; Morgan, 1990). Others have adopted solvent-free methods in which the insect cuticular surface is rubbed against adsorptive materials like solid-phase microextraction (SPME) fibers that can be directly inserted into the GC injector for desorption (Moneti et al., 1997; Tentschert et al., 2002). Turillazzi et al. (1998) rubbed the cuticular surface of live insects with a clean piece of cotton wool, which was then washed using organic solvents to recover the surface CHCs. Roux et al. (2009) proposed a water-based technique, in which CHCs were extracted from live insects by placing them into glass vials half-filled with warm water (≈ 34°C) that were vigorously shaken to form an emulsion. After removing the insects, the CHCs were recovered from the emulsion by extracting it with hexane.

Due to their ease of use and efficacy, solvent-soaking and SPME fiber extraction methods are commonly used to study insect CHCs. However, both methods have advantages and disadvantages. For example, solvent-soaking methods may extract compounds that are typically not accessible to the olfactory or gustatory organs of other insects, including internal body lipids and exocrine gland secretions, which may thus "contaminate" the CHC extracts (Monnin et al., 1998; Vander Meer and Morel, 1998; Lacey et al., 2008; Ginzel, 2010). On the other hand, SPME is likely to extract only those chemicals that are potentially accessible to the olfactory and gustatory organs of other insects (Ginzel et al., 2003, 2006). In addition, extraction with SPME fibers is less invasive than solvent-soaking, and insects are likely to survive the extraction procedure, thus allowing sequential examination of chemical profiles across developmental stages (Monnin et al., 1998). Nonetheless, SPME fiber extraction methods have their own limitations. First, SPME fibers extract additional non-CHC compounds along with other CHCs that may co-elute with the CHCs of interest during GC analysis (Tentschert et al., 2002). Second, SPME fibers are expensive and several fibers are required to collect multiple samples simultaneously. Third, SPME fibers require the use of mechanical pressure against the insect's cuticle, thus their use on small and/or fragile insects may not be possible (Turillazzi et al., 1998; Roux et al., 2009). Fourth, samples collected using SPME fibers cannot be stored for extended periods of time, and thus a GC instrument must be readily available (Turillazzi et al., 1998; Roux et al., 2009). Fifth, the compounds extracted via SPME fibers will only include those that are present in the specific area that was rubbed (e.g., thorax, abdomen, or legs) (Liebig et al., 2000; Lenoir et al., 2009). Because some insects have quantitatively different CHC profiles distributed throughout the body (Bagnères and Morgan, 1990; Bonavita-Cougourdan et al., 1993; Lenoir et al., 2009), SPME-based extraction may not include a representative sampling of the overall CHC profiles of the whole body surface. Finally, SPME techniques yield minute quantities of material, typically enough for analysis by GC, CG-MS, and possibly GC-FTIR, but the quantity of extracted compounds is rarely sufficient for behavioral assays (Millar and Sims, 1998).

Here, we describe a novel extraction technique for the analysis of insect CHCs. Silica gel possesses excellent adsorptive properties, which are ideal for extracting a wide range of compound classes. Fine-granule particles of silica gel (a granular, vitreous, highly porous form of silicon dioxide) and diatomaceous earth (composed of >90% silicon dioxide) have been used as insecticidal agents for their capacity of removing lipid layers from insect cuticles primarily by adsorption mechanisms (Ebeling, 1961, 1971; Cook et al., 2008). If insects contact these adsorptive dust particles, they lose a significant portion of their lipid layer from the cuticular surface, and thus become susceptible to rapid desiccation. Because silica dust particles are chemically stable and clean, it is possible to use organic solvents to recover these adsorbed lipid compounds for subsequent analyses. For example, Cook et al. (2008) successfully recovered cuticular compounds of mites (hydrocarbons and fatty acids) for GC-MS analysis by extracting diatomaceous earth that had been exposed to the mites for several hours. Chen (2007) reported that CHCs and other venom alkaloids were isolated from silica gel powder that was used as the nesting material by the red imported fire ant, *Solenopsis invicta* Buren. In the present study, we test whether CHCs can be extracted from insects using a novel extraction method: "silica-rubbing". The CHCs are first adsorbed onto the silica gel particles, and are then selectively eluted with a nonpolar solvent for subsequent GC-MS analysis. We validate the silica-rubbing method by comparing it with other two common extraction methods: solvent extraction and SPME.

Methods and Materials

Insects Chemical extractions were conducted using four species of ants and one species of bee. For the ant species, we used workers of the Argentine ant [*Linepithema humile* (Mayr) (Dolichoderinae)], *Azteca instabilis* (Smith) (Dolichoderinae),

the Florida carpenter ant (*Camponotus floridanus* (Buckley)) (Formicinae), and the red harvester ant [*Pogonomyrmex barbatus* (Smith) (Myrmicinae) (Hymenoptera: Formicidae)]. We used males of the solitary / semi-social orchid bee, *Euglossa dilemma* Eltz & Bembé (Hymenoptera: Apidae). The workers of *L. humile* were obtained from an outdoor foraging trail at Berkeley, CA in October 2010. Workers of *A. instabilis* were collected at the Finca Irlanda, Chiapas, Mexico in July 2010 (K. A. Mathis, University of California, Berkeley). The workers of *C. floridanus* were obtained from a laboratory colony collected at the Archbold Biological Station, FL in August 2009. The workers of *P. barbatus* were obtained from a laboratory colony collected in the field near Rodeo, NM in 2006 (S. Sturgis, Stanford University, Palo Alto). The colonies of *L. humile, A. instabilis,* and *C. floridanus* were fed with 25% (wt/vol) sucrose water, protein solution, and scrambled eggs three times a week. The colony of *P. barbatus* was provided with water, seeds (Wild Bird Food, Priority Total Pet Care, Pleasanton), apples, and crickets *ad libitum*. All ant colonies were maintained at room temperature. Males of the orchid bee *E. dilemma* (Eltz et al., 2011) were collected at chemical baits in Ft. Lauderdale, FL in February 2011, and kept in a temperature and humidity controlled insectary room at the University of California, Berkeley (see Ramírez et al., 2010 for details).

Extraction by Silica-Rubbing Live insects were anesthetized with CO_2 and subsequently killed by freezing in dry ice for 1 min. We placed the freeze-killed specimen under a fume hood for 5–10 min to remove any moisture that may have condensed on the surface of specimens while thawing. Surface lipids were extracted by placing the thawed insects in a 2-ml glass vial or small test tubes (10×75 or 13×100 mm) with 0.1~0.15 g of silica gel (70–230 mesh, Fisher Scientific), and subsequently vortexing it for 30 s. The silica gel in the vial or test tube was previously washed with 300–500 μl of hexane and dried under constant N_2 flow until individual gel particles moved freely without clumping. We extracted from single individuals, except for *L. humile*, for which we used a total of 30 ants per extraction. After vortexing, the insects were carefully removed with clean forceps, and the silica gel was extracted with hexane. The dried (with Na_2SO_4) silica extracts were subjected to flash liquid chromatography (0.4-cm diam×1.5-cm long column packed with 70–230 mesh silica gel) by elution with hexane. The volume of hexane used for the extraction and elution varied among species depending on the amount of silica gel used for initial extraction (Table 1). Both *A. instabilis* and *C. floridanus* have polymorphic workers, and thus only major workers were used for extraction.

Extraction by Hexane-Soaking Live insects were anesthetized using CO_2 and subsequently killed by freezing on dry ice for 1 min. Cuticular lipids were extracted by soaking the thawed insects in hexane for 10 min. A single insect was used in each replicated extraction, except for *L. humile*, for which we used a total of 30 ants per extraction in order to obtain enough CHCs for subsequent GC-MS analysis. The dried extracts were subjected to flash liquid chromatography by elution with hexane. The volume of hexane used for the extraction and elution varied among species, depending on the amount of hexane required to completely submerge the insects (Table 1).

Solid-Phase Microextraction (SPME) Using the same individuals of male *E. dilemma,* we compared the chemical profiles obtained via SPME with those obtained via silica-rubbing and hexane-soaking techniques. *Euglossa dilemma* was chosen because of its relatively large body size. A single SPME fiber (Supelco Inc.) coated with a 65-μm polydimethylsiloxane / divinylbenzene (PDMS / DVB) stationary phase was rubbed on the cuticle of an anaesthetized (CO_2) bee (three times on both the thoracic and the gastric tergites). The fiber was immediately injected into a GC-MS. We first extracted each individual bee using SPME, and subsequently applied the silica-rubbing and solvent-soaking techniques, in that order.

Efficacy of Silica-Rubbing To determine the efficacy of silica-rubbing in removing the cuticular lipid layer, we compared the rates of water loss of extracted and non-extracted workers of *L. humile* and *C. floridanus.* The rate of water loss was estimated by measuring weight loss over time under ambient conditions [20–28°C, 22–34% relative humidity (RH)]. A group of insects killed by freezing were subjected to silica-rubbing. Another group of insects was killed by freezing but not subjected to extraction, which thus served as control. To determine rates of water loss, the weights of extracted and control insects were measured immediately after extraction and 5 h (for *L. humile*) or 24 h (for *C. floridanus*) after extraction. An individual worker (*C. floridanus*) and a group of 30 workers (*L. humile*) were used to obtain each replicate measurement.

In *E. dilemma*, the efficacy of silica-rubbing was determined by comparing the total amount of CHCs extracted (via hexane-soaking) from insects that had been previously extracted by silica-rubbing to the amount of CHCs recovered by hexane-soaking without prior silica-rubbing. The CHC extracts were prepared using the method previously described in hexane-soaking. Because volumes of the extracts examined via GC-MS (1 μl) were identical throughout the GC-MS analysis, we directly compared the total integrated peak areas of the selected CHCs between the insects previously extracted via silica-rubbing and non-extracted controls.

Table 1 Amounts of hexane used for initial extraction and elution of hydrocarbons during flash liquid chromatography

Method		*L. humile*	*A. instabilis*	*C. floridanus*	*P. barbatus*	*E. dilemma*
Silica-rubbing	Extraction	200 µl	200 µl	300 µl	200 µl	1 ml
	Elution	200 µl	200 µl	100 µl	200 µl	-
Hexane-soaking	Extraction	200 µl	200 µl	200 µl	300 µl	1 ml
	Elution	200 µl	200 µl	300 µl	200 µl	1 ml

Chemical Analyses Prior to conducting GC-MS analyses, all CHC extracts were examined viathin-layer chromatography (TLC) for purity and concentration. TLC plates were developed with 100% hexane, and spots were visualized by spraying 5% (wt/vol) phosphomolybdic acid in ethanol, followed by heating with a heat gun. The concentration of CHC extracts was estimated based on the intensity of the dark spots. When needed, samples obtained via either silica-rubbing or hexane-soaking were concentrated under constant N_2 flow or diluted by adding clean hexane to make within-species concentrations similar. To minimize the loss of target compounds through volatilization, most samples were analyzed via GC-MS immediately after preparation, but some were stored in sealed vials at −20°C for<24 h prior to the analysis. For GC-MS, electron impact mass spectra (70 eV) were acquired with an Agilent 5975 C mass selective detector interfaced to a Agilent 7890A gas chromatograph fitted with an DB-5 column (30-m×0.32-mm i.d., Agilent Technologies). Extracts were analyzed in a splitless mode, with a temperature program that started at 100°C for 1 min which then increased by 15°C min^{-1} until it reached 300°C. Injector and transfer line temperatures were kept at 300°C (250°C for SPME) and 280°C, respectively. Individual hydrocarbon peaks were identified by comparing retention times and mass spectra with those of synthetic standards, matching with previously published spectra, and studying fragmentation patterns.

Statistical Analyses Automatic peak integration of chromatograms was conducted using the software Chemstation vE.02.00 (Agilent Technologies). We selected major peaks that were consistently present across samples on each species. Minor peaks with inconsistent integration results were excluded from the analyses. For each individual, CHC profiles were quantified by dividing the peak area of each compound by the total area of peaks selected for the analysis. The relative proportions of individual peaks were compared across extraction methods using Wilcoxon rank-sum test or Kruskal-Wallis ANOVA. To compare CHC profiles within and between different extraction methods, the relative areas of the selected CHC peaks were subjected to the non-metric Multidimensional Scaling (nMDS), an ordination technique where a predetermined number of axes of variation are chosen, and non-metric distances are fitted to those dimensions. We calculated a triangular distance matrix between samples (individuals) using the Bray-Curtis index of dissimilarity. We computed 2-dimensional MDS plots (50 iterations per run) using the software package *ECODIST v1.2.2* (written in R). We ran each analysis 10 times, and visually checked for convergence between solutions. To statistically assess whether CHC profiles exhibit greater dissimilarity between methods of extraction than within methods of extraction, we conducted Analysis of Similarity (ANOSIM) tests, as implemented in the software package *VEGAN v1.15-4* (written in R). We also estimated the relative contribution of individual compounds to the observed ordinal dissimilarities using the the Similarity Percentage (SIMPER) method, as implemented in the software package *PRIMER v6* (Clarke and Gorley, 2006). All other calculations, plots, and statistical tests were performed using basic R packages (http://cran.r-project.org).

Results

Linepithema humile Representative chromatograms of CHCs obtained with silica-rubbing and hexane-soaking are shown in Fig. 1a, with compounds numbered in order of elution (retention time). Thirteen hydrocarbons (saturated and methyl-branched alkanes) with chain lengths ranging from C27 to C37 were selected for comparisons between extraction methods (Table 2). The total areas of thirteen CHC peaks obtained by silica-rubbing and hexane-soaking were $(13.2\pm3.4)\times10^7$ and $(8.5\pm1.0)\times10^7$, respectively (mean±SEM, N=9 for silica-rubbing and N=10 for hexane-soaking).

The two different methods of extraction produced qualitatively similar GC-MS profiles for *L. humile*. However, quantitative differences were observed in some hydrocarbons (Fig. 1b and c; Table 2). The most conspicuous differences between extraction methods were the significantly greater proportions of three saturated alkanes (n-C_{27}, n-C_{29}, and n-C_{32}) in silica-rubbing extracts, and greater proportions of three methyl-branched alkanes (dimethyl-C_{35}, trimethyl-C_{35}, and trimethyl-C_{37}) in hexane-soaking extracts (Fig. 1c; Table 2).

The nMDS analysis showed a strong central tendency separation by extraction method (Fig. 1d). This pattern was further supported by the ANOSIM results, where a greater dissimilarity among CHC profiles was found between extraction methods than within them (R=0.419, P=0.001).

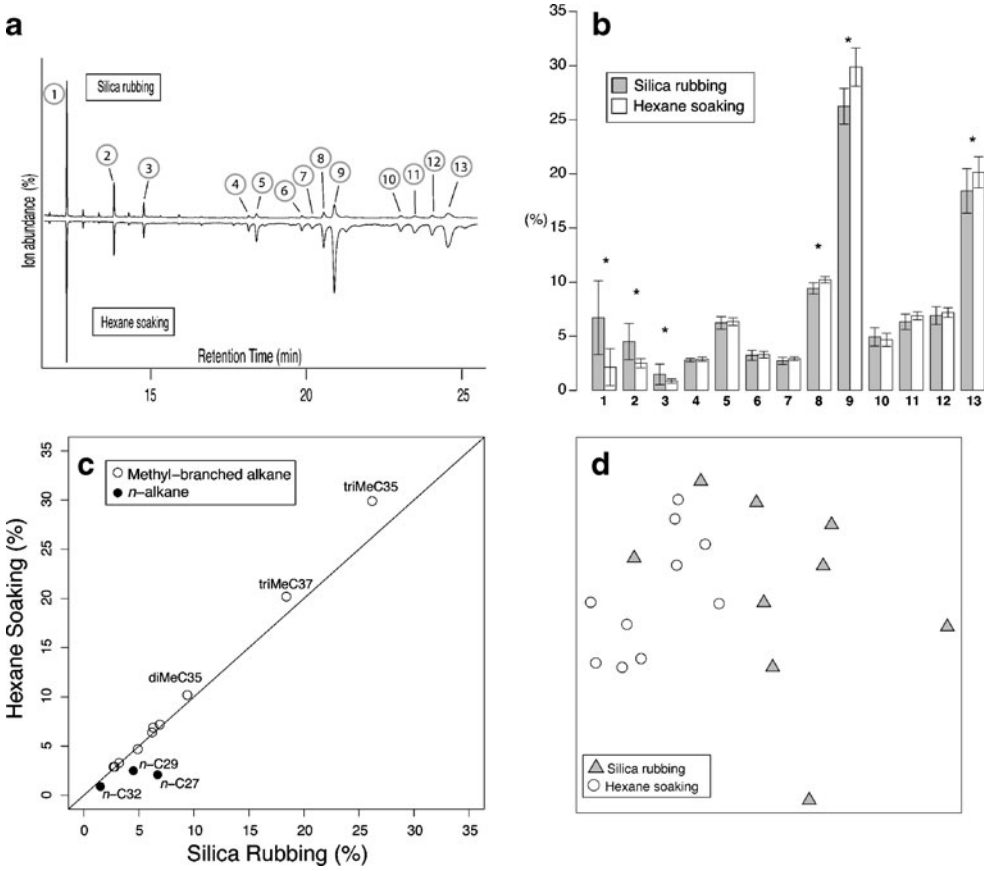

Fig. 1 Analyses of cuticular hydrocarbons (CHCs) from workers of *Linepithema humile*. (**a**) Representative total ion chromatograms of CHCs extracted by silica-rubbing (top) and hexane-soaking (bottom). (**b**) Proportional abundance (mean±SD) of the selected peaks. Bars with asterisk indicate that the proportional areas of the peak were significantly different between two extraction methods (Wilcoxon rank-rum test: α=0.05). (**c**) Scatter plot of proportional abundances (mean) of the selected peaks in silica-rubbing versus hexane-soaking. The sold line is the 45° perfect fit reference. (**d**) Separation of two different extraction methods based on non-metric Multidimensional Scaling (nMDS) of relative proportions of the selected peaks

The SIMPER analysis revealed that three peaks (peaks 1, 9, and 13) jointly contributed to >60% of the observed chemical dissimilarity between silica-rubbing and hexane-soaking extracts.

Table 2 Cuticular hydrocarbons of *Linepithema humile* and their relative proportions

Peak No.	Components	Composition (%, mean±SD)[a]	
		Silica-rubbing	Hexane-soaking
1	*n*-C_{27}	6.7±3.4*	2.1±1.7
2	*n*-C_{29}	4.5±1.7*	2.5±0.4
3	*n*-C_{32}	1.5±1.0*	0.9±0.2
4	5,15- and 5,17-diMeC_{33}	2.8±0.2	2.9±0.2
5	5,15,19-triMeC_{33}	6.2±0.6	6.4±0.4
6	MeC_{35}	3.2±0.5	3.3±0.3
7	15,19-diMeC_{35}	2.7±0.3	2.9±0.2
8	5,15- and 5,17-diMeC_{35}	9.4±0.5	10.2±0.3*
9	5,13,17- and 5,15, 19-triMeC_{35}	26.2±1.7	29.9±1.8*
10	13- and 15- and 17- and 19-MeC_{37}	4.9±0.8	4.7±0.6
11	15,19-diMeC_{37}	6.3±0.7	6.9±0.4
12	5,15- and 5,17-diMeC_{37}	6.9±0.82	7.2±0.4
13	5,15,19- and 5,13, 17-triMeC_{37}	18.4±2.1	20.2±1.4*

[a] Value with asterisk is significantly larger than a corresponding value in the other extraction method (Wilcoxon rank-sum test: α=0.05)

Azteca instabilis Representative chromatograms of CHCs obtained via silica-rubbing and hexane-soaking are shown in Fig. 2a. Thirteen hydrocarbons (saturated and methyl-branched alkanes) with chain lengths ranging from C23 to C29 were selected to compare the different extraction methods (Table 3). The total areas of thirteen CHC peaks obtained by silica-rubbing and hexane-soaking were $(13.1\pm1.6)\times10^8$ and $(8.0\pm0.6)\times10^8$, respectively (mean±SEM, N=10 for each treatment).

The two different methods of extraction produced qualitatively similar GC-MS profiles for *A. instabilis*. However, we found substantial quantitative differences for most hydrocarbons (Fig. 2b and c; Table 3). The most conspicuous differences between extraction methods were the significantly greater proportion of most methyl-branched alkanes (monomethyl and dimethyl alkanes) in silica-rubbing extracts, and the greater proportion of three saturated alkanes (*n*-C_{25}, *n*-C_{26}, and *n*-C_{27}) and a single methyl-branched alkane (3-MeC_{27}) in hexane-soaking extracts (Fig. 2c; Table 3).

The nMDS analysis showed a strong clustering by extraction method (Fig. 2d). This pattern was further supported by the ANOSIM analysis, suggesting a greater dissimilarity

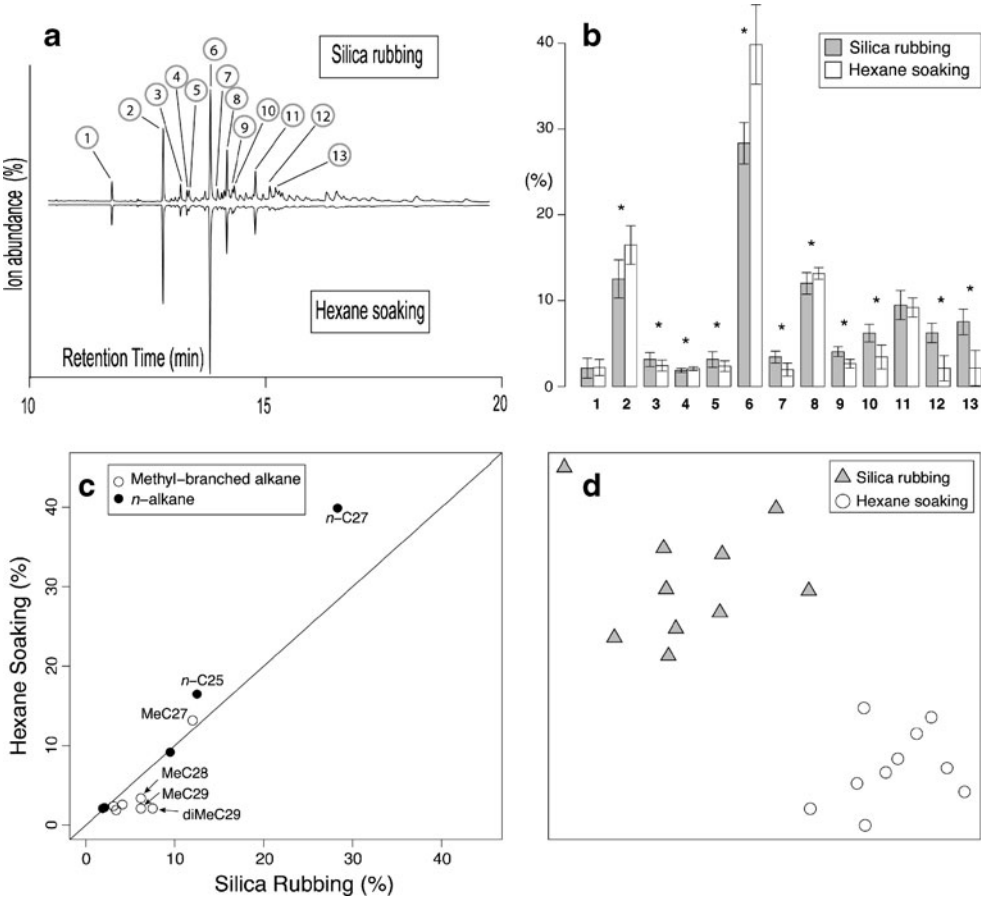

Fig. 2 Analyses of cuticular hydrocarbons (CHCs) from workers of *Azteca instabilis*. (**a**) Representative total ion chromatograms of CHCs extracted by silica-rubbing (top) and hexane-soaking (bottom). (**b**) Proportional abundance (mean±SD) of the selected peaks. Bars with asterisk indicate that the proportional areas of the peak were significantly different between two extraction methods (Wilcoxon rank-rum test: α=0.05). (**c**) Scatter plot of proportional abundances (mean) of the selected peaks in silica-rubbing versus hexane-soaking. The sold line is the 45° perfect fit reference. (**d**) Separation of two different extraction methods based on non-metric Multidimensional Scaling (nMDS) of relative proportions of the selected peaks

Table 3 Cuticular hydrocarbons of *Azteca instabilis* and their relative proportions

Peak No.	Components	Composition (%, mean±SD)[a]	
		Silica-rubbing	Hexane-soaking
1	*n*-C_{23}	2.1±1.2	2.2±0.9
2	*n*-C_{25}	12.5±2.2	16.5±2.2*
3	3-MeC_{25}	3.1±0.8*	2.4±0.6
4	*n*-C_{26}	1.9±0.2	2.1±0.2*
5	10- and 12- and 14-MeC_{26}	3.1±0.9*	2.4±0.6
6	*n*-C_{27}	28.3±2.4	39.9±4.6*
7	11- and 13-MeC_{27}	3.4±0.7*	1.9±0.8
8	3-MeC_{27}	12.0±1.3	13.2±0.7*
9	6,16- and 8,15-diMeC_{27}	4.1±0.6*	2.6±0.5
10	10- and 12- and 13- and 14-MeC_{28}	6.2±1.0*	3.4±1.4
11	*n*-C_{29}	9.5±1.7	9.2±1.1
12	MeC_{29}	6.2±1.1*	2.1±1.5
13	7,15- and 7,17-diMeC_{29}	7.5±1.5*	2.1±2.1

[a] Value with asterisk is significantly larger than a corresponding value in the other extraction method (Wilcoxon rank-sum test: α=0.05)

of CHC profiles between extraction methods than within them (R=0.979, P=0.001). The SIMPER analysis revealed that three peaks (peaks 6, 12, and 13) jointly contributed to >50% of the observed chemical dissimilarity between silica-rubbing and hexane-soaking extracts.

Pogonomyrmex barbatus Representative chromatograms of CHCs obtained by silica-rubbing and hexane-soaking are shown in Fig. 3a. Ten hydrocarbons (saturated and methyl-branched alkanes) with chain lengths ranging from C23 to C31 were selected for the comparison between extraction methods (Table 4). The total areas of ten CHC peaks obtained via silica-rubbing and hexane-soaking were (2.0±0.3)×10^7 and (2.3±0.2)×10^7, respectively (mean±SEM, N=10 for each treatment).

The two different methods of extraction produced qualitatively similar, but quantitatively different GC-MS profiles. Proportional differences were observed in 9 of the 10 hydrocarbons selected for the analysis (Fig. 3b and c; Table 4). Seven peaks of methyl-branched alkanes (monomethyl and dimethyl alkanes) were found in greater proportions in silica-rubbing extracts. On the other hand, two saturated alkanes (*n*-C_{25} and *n*-C_{27}) were found in greater proportions in hexane-soaking extracts (Fig. 3c; Table 4).

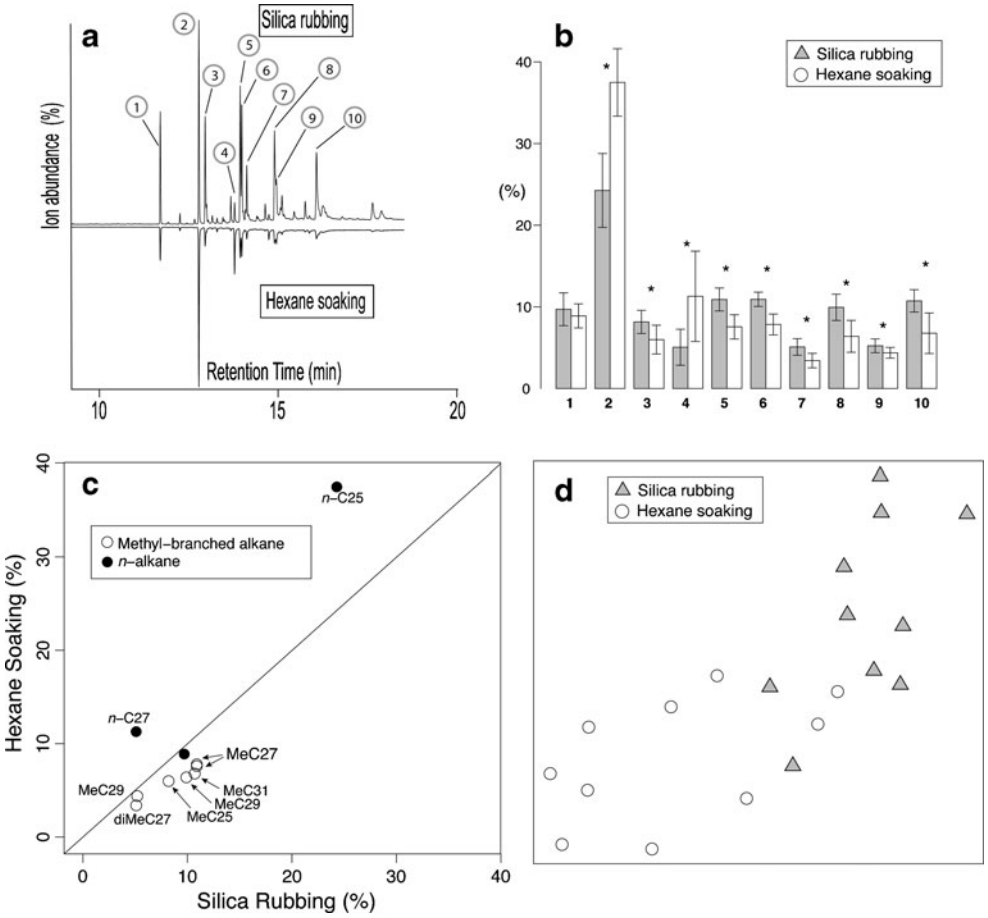

Fig. 3 Analyses of cuticular hydrocarbons (CHCs) from workers of *Pogonomyrmex barbatus*. (**a**) Representative total ion chromatograms of CHCs extracted by silica-rubbing (top) and hexane-soaking (bottom). (**b**) Proportional abundance (mean±SD) of the selected peaks. Bars with asterisk indicate that the proportional areas of the peak were significantly different between two extraction methods (Wilcoxon rank-rum test: α=0.05). (**c**) Scatter plot of proportional abundances (mean) of the selected peaks in silica-rubbing versus hexane-soaking. The sold line is the 45° perfect fit reference. (**d**) Separation of two different extraction methods based on non-metric Multidimensional Scaling (nMDS) of relative proportions of the selected peaks

The nMDS analysis indicated a strong clustering by extraction method (Fig. 3d). This pattern was further supported by the ANOSIM analysis, where a greater dissimilarity among CHC profiles was found between extraction methods than within them (R=0.7, P=0.001). The SIMPER analysis revealed that a single compound (peak 2: n-C_{25}) contributed to >50% of the observed chemical dissimilarity between silica-rubbing and hexane-soaking extracts.

Table 4 Cuticular hydrocarbons of *Pogonomyrmex barbatus* and their relative proportions

Peak No.	Components	Composition (%, mean±SD)[a]	
		Silica-rubbing	Hexane-soaking
1	n-C_{23}	9.7±2.0	8.9±1.5
2	n-C_{25}	24.3±4.5	37.5±4.1*
3	13-MeC_{25}	8.2±1.4*	6.0±1.8
4	n-C_{27}	5.1±2.2	11.3±5.5*
5	13-MeC_{27}	10.9±1.4*	7.6±1.5
6	7-MeC_{27}	10.9±0.9*	7.8±1.3
7	7,13-diMeC_{27}	5.1±1.0*	3.4±0.9
8	15-MeC_{29}	9.9±1.6*	6.4±2.0
9	7- and 9-MeC_{29}	5.2±0.8*	4.4±0.7
10	11- and 13- and 15-MeC_{31}	10.7±1.4*	6.8±2.5

[a] Value with underline is significantly larger than a corresponding value in the other extraction method (Wilcoxon rank-sum test: α=0.05)

Camponotus floridanus Representative chromatograms of CHCs obtained by silica-rubbing and hexane-soaking are shown in Fig. 4a. Eighteen hydrocarbons (saturated and methyl-branched alkanes) with chain lengths ranging from C30 to C33 were selected for comparison between extraction methods (Table 5). The total areas of eighteen CHC peaks obtained via silica-rubbing and hexane-soaking were $(10.5\pm0.9)\times10^8$ and $(11.0\pm1.3)\times10^8$, respectively (mean± SEM, N=10 for each treatment).

Even if two different extraction methods produced qualitatively similar GC-MS profiles, we found quantitative differences in 5 of 18 hydrocarbons (Fig. 4b and c; Table 5). The most conspicuous differences between extraction methods were the significantly greater proportions of three peaks of methyl-branched alkanes (dimethyl and trimethyl alkanes) in silica-rubbing extracts, and greater proportions of one saturated alkane (n-C_{31}) and three co-eluting dimethylhentriacontanes (5,9- and 5,11- and 5,13-diMeC_{31}) in hexane-soaking extracts (Fig. 4c; Table 5).

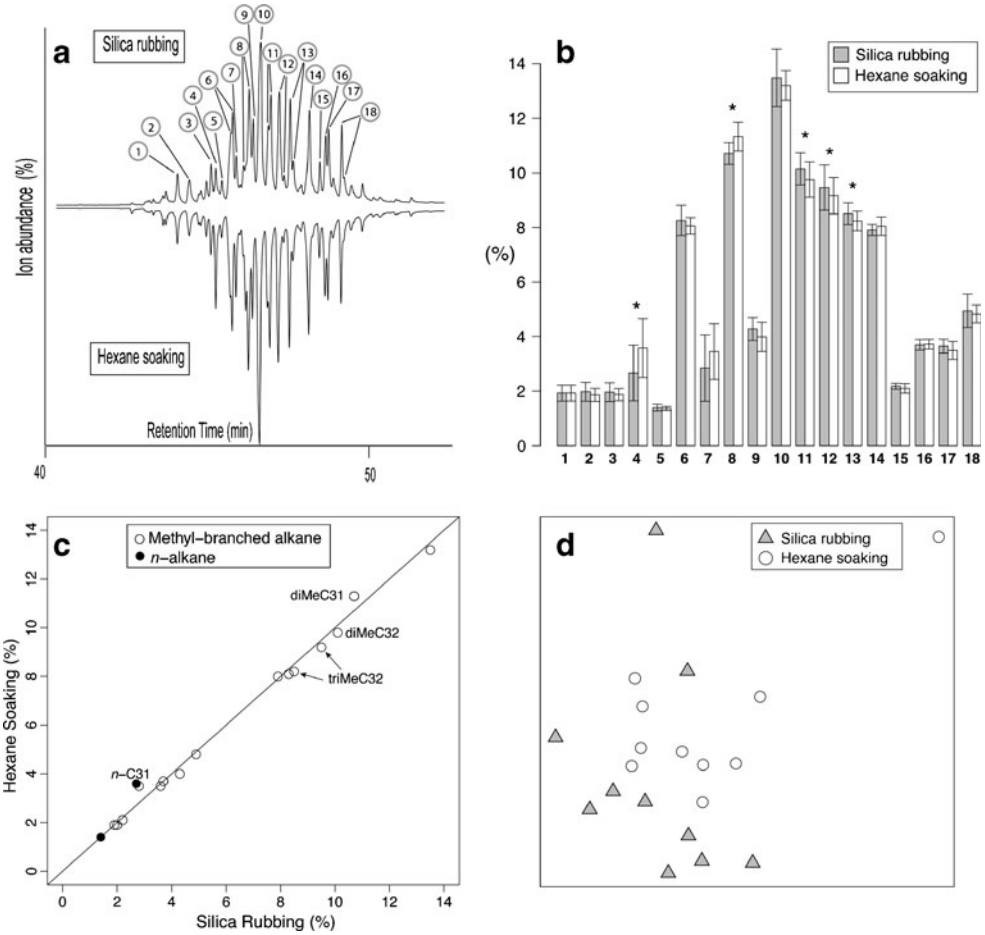

Fig. 4 Analyses of cuticular hydrocarbons (CHCs) from workers of *Camponotus floridanus*. (**a**) Representative total ion chromatograms of CHCs extracted by silica-rubbing (top) and hexane-soaking (bottom). (**b**) Proportional abundance (mean ±SD) of the selected peaks. Bars with asterisk indicate that the proportional areas of the peak were significantly different between two extraction methods (Wilcoxon rank-rum test: α=0.05). (**c**) Scatter plot of proportional abundances (mean) of the selected peaks in silica-rubbing versus hexane-soaking. The sold line is the 45° perfect fit reference. (**d**) Separation of two different extraction methods based on non-metric Multidimensional Scaling (nMDS) of relative proportions of the selected peaks

The nMDS analysis revealed a moderate clustering by extraction method (Fig. 4d). Likewise, the ANOSIM analysis indicated a marginally greater dissimilarity of CHC profiles between extraction methods than within them (R=0.2, P=0.014). The SIMPER analysis revealed that five peaks (peaks 4, 7, 8, 10, and 12) jointly contributed to >50% of the observed chemical dissimilarity between silica-rubbing and hexane-soaking extracts.

Euglossa dilemma Representative chromatograms of CHCs obtained by SPME, silica-rubbing, and hexane-soaking are shown in Fig. 5a. Five hydrocarbons (three *n*-alkanes and two alkenes) with chain lengths ranging from C23 to C27 were selected to compare extraction methods (Table 6). The total areas of five CHC peaks obtained via SPME, silica-rubbing, and hexane-soaking were $(12.0\pm1.7)\times10^7$, $(8.9\pm0.6)\times10^7$, and $(6.2\pm0.4)\times10^7$, respectively (mean±SEM, N=9 for SPME and N=10 for the rest).

Three different methods of extraction (i.e., SPME, silica-rubbing, and hexane-soaking) produced qualitatively similar but quantitatively different GC-MS profiles for three of five hydrocarbons (Fig. 5b and c; Table 6). Two alkene compounds (C_{25}:1 and C_{27}:1) were present in greater proportions in hexane-soaking extracts than either in SPME or silica-rubbing extracts (Fig. 5b and c; Table 6). However, one saturated alkane (n-C_{27}) was found in smaller proportions in the hexane-soaking extracts compared to either SPME or silica-rubbing extracts (Fig. 5b and c; Table 6).

The nMDS analysis showed a strong clustering by extraction method (Fig. 5d). This pattern was supported by the ANOSIM analysis, which showed a greater dissimilarity of CHC profiles between methods than within them (R=0.4, P=0.001). The SIMPER analysis revealed that two peaks (peaks 2 and 4 for SPME vs. silica-rubbing; peaks 3 and 4 for SPME vs. hexane-soaking and silica-rubbing vs. hexane-soaking) jointly contributed >50% of the observed chemical dissimilarity between two different extraction methods.

Efficacy of Silica-Rubbing The insects extracted using the silica-rubbing method lost more water via evaporation than did the non-extracted control specimens. During the first 5 h after the extraction was conducted, the *L. humile* workers lost 62.7±0.4% (mean±SEM, N=5) of their body weight, whereas the control ants lost 20.8±0.5% (N=3) (two-sample t-test: t=65.5, df=6, P<0.001). The initial weights of the extracted and control insects were 11.3±0.1 and 12.8±0.2 mg, respectively (mean±SEM). In the case of *C. floridanus*, the extracted worker ants lost 51.3±1.9% of their body weight compared to

Table 5 Cuticular hydrocarbons of *Camponotus floridanus* and their relative proportions

Peak No.	Components	Composition (%, mean±SD)[a]	
		Silica-rubbing	Hexane-soaking
1	10- and 12- and 14-MeC_{30}	1.9±0.3	1.9±0.3
2	8,16- and 10,16-diMeC_{30}	2.0±0.3	1.9±0.2
3	Methyl-branched C_{30}	2.0±0.8	1.9±0.2
4	*n*-C_{31}	2.7±1.0	3.6±1.1*
5	Methyl-branched C_{31}	1.4±0.1	1.4±0.1
6	7- and 9- and 11- and 13-MeC_{31}	8.3±0.6	8.1±0.3
7	5-MeC_{31}	2.8±1.2	3.5±1.0
8	5,9- and 5,11- and 5, 13-diMeC_{31}	10.7±0.4	11.3±0.5*
9	7, 11, 15-triMeC_{31}	4.3±0.4	4.0±0.5
10	10- and 12-MeC_{32}	13.5±1.1	13.2±0.5
11	8,12- and 10,14-diMeC_{32}	10.1±0.6*	9.8±0.6
12	5,9,13-triMeC_{32}	9.5±0.8*	9.2±0.7
13	8,12,16-triMeC_{32}	8.5±0.4*	8.2 ±0.4
14	4,8,12,16-tetraMeC_{32}	7.9±0.2	8.0±0.3
15	7,11-diMeC_{33}	2.2±0.1	2.1±0.2
16	5,9- and 5,11- and 5, 13-diMeC_{33}	3.7±0.2	3.7±0.2
17	7,11,15-triMeC_{33}	3.6±0.3	3.5±0.3
18	5,9,13,17-tetraMeC_{33}	4.9±0.6	4.8±0.3

[a] Value with asterisk is significantly larger than a corresponding value in the other extraction method (Wilcoxon rank-sum test: α=0.05).

5.3±1.2% in the control ants (mean±SEM, N=10 for each treatment) during the first 24 h after the extraction (two-sample t-test: t=20.8, df=18, P<0.001). The initial weights of the extracted and control insects were 26.6±1.7 and 27.7±1.5 mg, respectively (mean±SEM).

Study with *E. dilemma* also indicated that silica-rubbing technique was effective in removing significant amount of CHCs from surface of the insects (i.e., > 50% of total amount extractable with solvent). The total peak area of five CHCs obtained by soaking the insects previously extracted via silica-rubbing [$(6.2\pm0.4)\times10^7$] was≈60% smaller than the total peak area of the CHCs obtained from control insects without previous extraction with silica-rubbing [$(15.6\pm1.4)\times10^7$] (mean±SEM, N=10 for each treatment) (Wilcoxon rank-sum test: z=3.7, P<0.001).

Discussion

Sampling of cuticular hydrocarbons (CHCs) by extracting dead insects with solvent has been an established method in chemical ecology of insects (Howard and Blomquist, 2005). However, such method has the disadvantage of extracting additional compounds from internal glands or deep wax layers that may not be readily available to olfactory or gustatory organs of other individual insects. The relatively recent introduction of SPME fibers alleviated this problem (Moneti et al., 1997), although it remains expensive and difficult to scale up for multiple individual extractions. Our study demonstrates that insect CHCs can be extracted quickly and easily using fine silica gel particles. Using a vortex mixer, cuticular surfaces of freeze-killed insects are rubbed with a small amount of granular silica gel. The adsorbed compounds are subsequently eluted with nonpolar organic solvents for further chemical analyses. Our results suggest that silica-rubbing is powerful enough to remove a significant portion of the lipid layer of insects.

The CHC profiles obtained via silica-rubbing and hexane-soaking were qualitatively similar, with all CHCs being present in extracts from both methods. However, the chemical profiles were quantitatively different. In particular, we found marked differences in the relative proportions of saturated alkanes, methyl-branched alkanes, and alkenes. For example, in the Argentine ant *L. humile* and the orchid bee *E. dilemma*, all or some of the saturated alkanes examined exhibited higher proportions in silica-rubbing samples than hexane-soaking samples. On the other hand, several methyl-branched alkanes (in *L. humile*) or alkenes (in *E. dilemma*) were relatively more abundant in hexane-soaking samples. In the other three ant species (*A. instabilis, P. barbatus,* and *C. floridanus*), the proportion of the most abundant saturated alkanes (i.e., *n*-C_{27}, *n*-C_{25}, and *n*-C_{31} for *A. instabilis*, *P. barbatus*, and *C. floridanus*, respectively) was consistently higher in hexane-soaking samples. On the other hand, several methyl-branched alkanes exhibited relatively higher concentrations in silica-rubbing samples.

Similar quantitative discrepancies between solvent extraction and SPME techniques have been previously reported in other insect species. For example, SPME samples of a cerambycid beetles cuticle contained greater proportions of methyl-branched alkanes and smaller proportions of saturated alkanes than the whole-body hexane extracts (Ginzel et al., 2003; Lacey et al., 2008). A female contact sex pheromone of a cerambycid beetle *Megacyllene robiniae*, (Z)-9-pentacosene, was present in hexane extracts of male insects, but represented in negligible proportions when the male insect cuticle was extracted using SPME fiber (Ginzel et al., 2003; Ginzel, 2010). Furthermore, in a queen of *Vespa* sp. wasp, the proportion of the most abundant saturated alkane was higher in hexane-soaking samples, whereas the proportion of the most abundant methyl-branched alkane was higher in SPME samples (Moneti et al., 1997). Monnin et al. (1998) noted that SPME sampling of ponerine ants underestimated the presence of shorter-chain hydrocarbons while overestimating the concentration of longer-chain hydrocarbons when it compared to pentane extracts of cuticle.

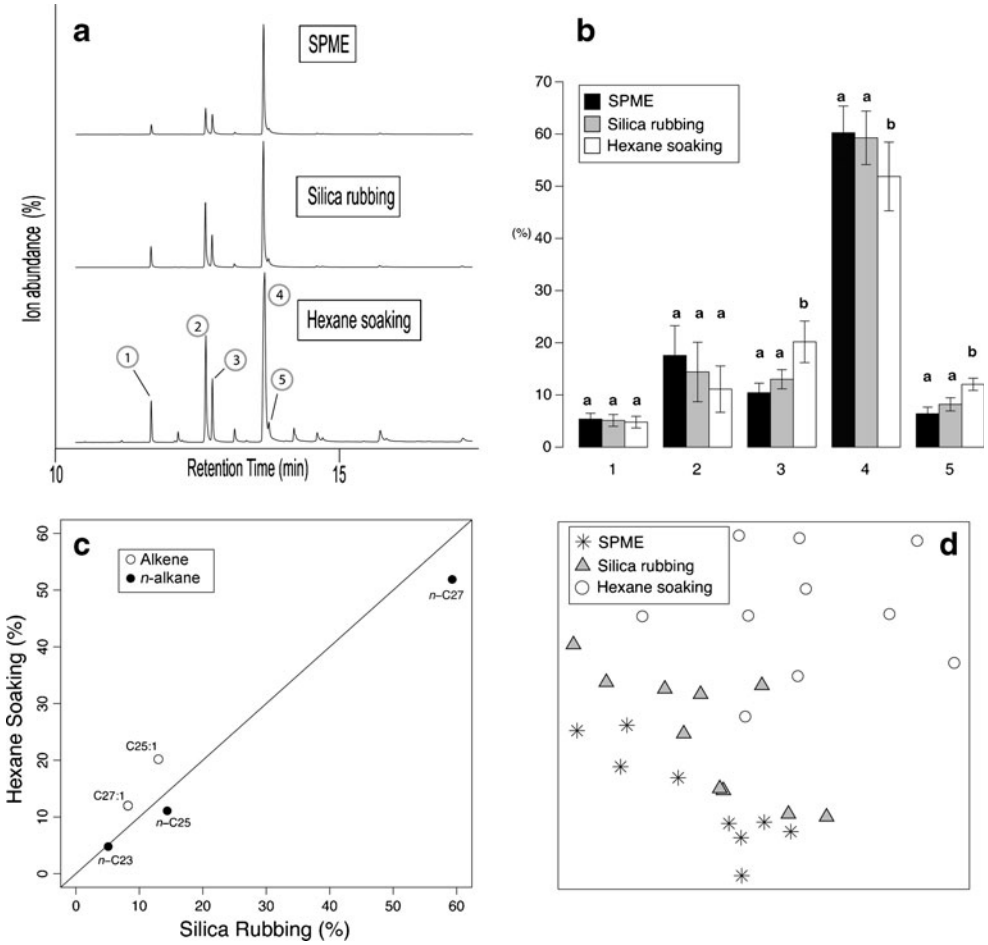

Fig. 5 Analyses of cuticular hydrocarbons (CHCs) from males of *Euglossa dilemma*. (**a**) Representative total ion chromatograms of CHCs extracted by SPME (top), silica-rubbing (middle), and hexane-soaking (bottom). (**b**) Proportional abundance (mean ±SD) of the selected peaks. Bars with the same letter within a peak are not significantly different (Kruskal-Wallis ANOVA followed by all-pairwise comparisons of mean ranks: α=0.05). (**c**) Scatter plot of proportional abundances (mean) of the selected peaks in silica-rubbing versus hexane-soaking. The sold line is the 45° perfect fit reference. (**d**) Separation of three different extraction methods based on non-metric Multidimensional Scaling (nMDS) of relative proportions of the selected peaks

Our study showed that the CHC profiles obtained via silica-rubbing and SPME fibers were more similar to each other than either one was to the profiles obtained via hexane-soaking (in *E. dilemma*). Thus, it is likely that the quantitative discrepancies between whole-body solvent extraction and surface wiping techniques result from the extraction of non-cuticular hydrocarbons (particularly the contents of internal glands) during whole-body solvent extraction. High selectivity is known to be involved in the synthesis, transportation, and deposition of insect hydrocarbons (Blomquist and Bagnères, 2010), resulting in different CHC profiles in different parts of body (e.g., cuticular body surface, glands, and alimentary tract). For example, Bagnères and Morgan (1991) reported substantial proportional differences between CHC profiles of cuticle and postpharyngeal gland of queen and worker *Myrmica rubra* L., where alkanes were relatively more abundant in the cuticle, and methyl-branched alkanes were more abundant in the gland. In some ants, colony-specific hydrocarbon mixtures are stored in the postpharyngeal gland, and lipids may be distributed from there throughout the body surface by grooming (Hefetz et al., 2001; Bagnères and Blomquist, 2010). Extraction of such glandular contents is likely to occur to a greater degree during solvent extraction than during silica-rubbing or SPME. Therefore, the silica-rubbing technique may be more appropriate than whole-body solvent extraction in studies that investigate the chemical ecology of cuticular substances.

Our study demonstrates that the silica-rubbing method can be effectively used as an alternative to SPME for sampling insect CHCs. Even though both methods target surface-

Table 6 Cuticular hydrocarbons of *Euglossa dilemma* and their relative proportions

Peak No.	Components	Composition (%, mean±SD)[a]		
		SPME[b]	Silica-rubbing[b]	Hexane-soaking[b]
1	*n*-C_{23}	5.4±0.8a	5.1±1.1a	4.8±1.1a
2	*n*-C_{25}	17.6±5.2a	14.4±5.7a	11.1±4.4a
3	C_{25}:1	10.4±1.2a	13.0±1.8a	20.2±4.0b
4	*n*-C_{27}	60.2±5.1a	59.3±5.1a	51.9±6.6b
5	C_{27}:1	6.4±0.7a	8.2±1.3a	12.0±1.2b

[a] Values followed by the same letter within each row are not significantly different (Kruskal-Wallis ANOVA followed by all-pairwise comparisons of mean ranks: α=0.05)

[b] Data were obtained from same individuals, which were extracted first by SPME and subsequently by silica-rubbing and hexane-soaking

specific compounds, silica-rubbing is advantageous because it allows extracting compounds from the entire body surface. Also, unlike SPME fibers, our silica gel method does not require the use of several extraction devices for simultaneously extracting multiple samples, and thus the total expenses are significantly reduced. Furthermore, extractions using silica-rubbing may permit the recovery of significant quantities of CHC that could be used in subsequent bioassays. Lastly, because only small quantities of non-toxic silica-gel are required, this technique is particularly suited for field studies, thus eliminating the need to transport highly flammable solvents. The silica-rubbing technique may yield a more representative CHC profile that is actually encountered by other insects' sensory organs, and thus it may facilitate the discovery and identification of biologically important components which are potentially meaningful in chemical communication. We suggest that silica-rubbing technique might be employed as a routine method for insect CHC analyses.

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Aliphatic Esters as Targets of Esterase Activity in the Parsnip Webworm (*Depressaria pastinacella*)

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Abstract As a specialist on the reproductive structures of *Pastinaca sativa* and species in the related genus *Heracleum*, the parsnip webworm *(Depressaria pastinacella*) routinely encounters a distinctive suite of phytochemicals in hostplant tissues. Little is known, however, about the detoxification mechanisms upon which this species relies to metabolize these compounds. In this study, larval guts containing hostplant tissues were homogenized, and metabolism was determined by incubating reactions with and without NADPH and analyzing for substrate disappearance and product appearance by gas chromatography–mass spectrometry. Using this approach, we found indications of carboxylesterase activity, in the form of appropriate alcohol metabolites for three aliphatic esters in hostplant tissues—octyl acetate, octyl butyrate, and hexyl butyrate. Involvement of webworm esterases in hostplant detoxification subsequently was confirmed with metabolism assays with pure compounds. This study is the first to implicate esterases in lepidopteran larval midgut metabolism of aliphatic esters, ubiquitous constituents of flowers and fruits. In addition, this method confirmed that webworms detoxify furanocoumarins and myristicin in their hostplants via cytochrome P450-mediated metabolism, and demonstrated that these enzymes also metabolize the coumarin osthol and the fatty acid derivative palmitolactone.

Keywords Aliphatic esters · Carboxylesterases · Cytochrome P450 · Furanocoumarin · Herbivore · Metabolic profiling · Osthol · Palmitolactone

Although this project was the brainchild of Arthur Zangerl, his untimely death prevented him from seeing this work in print. His quarter-century-long dedication to studying the interaction between parsnip webworms and wild parsnips was essential to making this system a model for chemical ecology studies.

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Introduction

In insects, three multigene families are substantially responsible for detoxification of xenobiotics: cytochrome P450 monooxygenases (P450), glutathione-S-transferases (GST), and carboxylesterases (Claudianos et al., 2006). In herbivorous insects, the P450s play a key role in detoxification of host phytochemicals (Li et al., 2007). Like several other lepidopteran species (*Papilio polyxenes*—Cohen et al., 1992, *Papilio glaucus*—Li et al., 2003; *Helicoverpa zea*—Li et al., 2004; *Papilio multicaudatus*—Mao et al., 2006; *Amyelois transitella*—Niu et al., 2011), the parsnip webworm *Depressaria pastinacella* relies on P450s to metabolize furanocoumarins encountered in tissues of its host plants in the apiaceous genera *Pastinaca* and *Heracleum* (Nitao et al., 2003; Mao et al., 2008). The extent to which other enzyme systems contribute to host phytochemical detoxification has not, however, been determined in this species.

As principal animal Phase II enzymes, most GSTs catalyze the conjugation of reduced glutathione with electrophilic (often lipophilic) substrates to form more water-soluble, non-toxic peptide derivatives that are excreted. Two classes of GSTs, Delta and Epsilon, are unique to insects, and several of these contribute to xenobiotic detoxification

(Ranson and Hemingway, 2005; Tu and Akgul, 2005). Gui et al. (2008) showed that one Delta and one Sigma class GST are expressed in silkworm midguts, suggesting a role in detoxification. Carboxylesterases constitute a gene superfamily that has seen an expansion in herbivorous insects. Tissue-specific patterns of expression of carboxylases in *Bombyx mori* suggest specific functions of subsets of these enzymes; those restricted to larval midguts likely contribute to host phytochemical metabolism (Yu et al., 2008, 2009). In the sole study to date directly demonstrating carboxylesterase-mediated metabolism of host phytochemicals, Lindroth (1989a,b) measured different esterase activity in the tiger swallowtails *Papilio glaucus* and *Papilio canadensis* (then considered subspecies), with *P. canadensis* displaying higher activity against the host plant phenolic glycosides typical of *P. canadensis* hosts.

In order to determine which detoxification systems may be involved in webworm adaptation to its hostplants, we utilized a screening method that exposes substrates of interest to all possible detoxification systems in the same assay. The conventional way to identify insect resistance mechanisms against plant chemical defenses, in place for many decades, has been to examine candidate metabolic pathways in individual bioassays with pure compounds. Although great strides have been made in this manner (e.g., cytochrome P450-mediated furanocoumarin metabolism by lepidopterans—*vide infra*), this approach is potentially biased by the availability of pure phytochemicals, failure to screen for interactions with co-occurring compounds, and the inherent narrow focus on specific metabolic pathways, leading to potential exclusion of alternative pathways that may contribute to detoxification.

An alternative approach is to expose an herbivore to the full range of potential substrates for detoxification in a particular host plant at ecologically relevant concentrations, and then to search for and identify metabolites. Metabolites of plant compounds can be screened inside the consumer as they are being processed or in the frass after processing has been completed. This approach enabled Salminen et al. (2004) to determine that the autumnal moth caterpillar (*Epirrita autumnata*) excretes flavonoid aglycones from birch leaves as glycosylated forms. Ferreres et al. (2009a, b) examined the bodies and frass of caterpillars of the large white butterfly caterpillar *Pieris brassicae* that had fed on *Brassica oleracea* and identified a number of metabolites that likely were derived from hostplant flavonoids, implicating deacylation, deglycosylation, and sulfate conjugation as detoxification pathways. The contributions of these enzyme systems to pierid utilization of host flavonoids, however, have not yet been confirmed. Jansen et al. (2009) utilized metabolic profiling to examine this same interaction and, by comparing metabolite profiles of both caterpillar and plant tissues, correlated levels of three coumaroylquinic acids in both species, and defined these compounds as the "metabolic interface," although the toxicological properties and detoxification pathways of these compounds remain unknown. In a more recent study, Glauser et al. (2011) utilized simultaneous metabolomics screening of living maize tissue and maize tissue digested by two *Spodoptera* species along with frass to demonstrate that the defensive plant metabolite 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one is not glycosylated by either caterpillar species, possibly explaining its significance as a defensive compound even against specialists.

To determine how *D. pastinacella* metabolizes potential toxins in its principal host plant *P. sativa* we developed a metabolic profiling assay that relies on homogenates from guts containing plant material consumed by the insect; this method exposes the full range of potential substrates for detoxification at ecologically relevant concentrations. Although precise phytochemical composition varies temporally and geographically, webworms in general encounter a distinctive suite of compounds that typify the genera *Pastinaca* and *Heracleum*. Among the specific compounds webworms reliably encounter when consuming plant reproductive tissue are aliphatic esters, coumarins, furanocoumarins, fatty acid derivatives, mono- and sesquiterpenes, and phenylpropanoids (Zangerl et al., 1997; Zangerl and Berenbaum, 2009). Because lepidopteran larvae deploy the bulk of their detoxification systems in the midgut, as the principal entry point for toxins, we created gut/food homogenates from guts filled by consumption of wild parsnip flowers. These homogenates require only a short incubation to reveal differential substrate disappearance. We also modified the incubation reactions, by addition of specific cofactors or inhibitors, to facilitate confirmation of the identity of the enzyme systems important to insect metabolism. In this way, we were able to confirm the contributions of cytochrome P450-mediated metabolism of furanocoumarins and myristicin (Nitao, 1989; Nitao et al., 2003; Mao et al., 2006, 2008) and also to identify for the first time carboxylesterase-mediated metabolism of aliphatic esters in this species.

Methods and Materials

Metabolic Profiling Experiment We designed this experiment to determine which of the non-polar constituents of host plants are metabolized by parsnip webworms. Two categories of metabolism were examined. Cytochrome P450 activity was determined by examining the difference between the amount of substrate remaining after gut homogenate incubation with NADPH present compared to the amount remaining after gut homogenate incubation in buffer only; NADPH is a cofactor that is essential for P450 metabolism (Li et al., 2007). Activity of metabolic pathways other than P450s was assessed by comparing substrate amounts remaining after incubation of gut homogenate in

plain buffer to the amount present before incubation, with substrate disappearance serving as an indicator of metabolism.

Twenty-two ultimate instar parsnip webworms from a colony maintained in our laboratory were reared from eggs on artificial diet containing no host plant (Nitao and Berenbaum, 1988). These larvae were transferred to containers containing flowers and developing fruits of wild parsnip from plants grown in a greenhouse from locally collected seed. After 2 d feeding on plant material, the larvae were dissected in 0.1 M, pH 7.8 phosphate buffer (21.42 g sucrose, 2.5 g PVP-10, 9.3 mg EDTA, and 87 mg PMSF per 250 ml buffer) on ice, and the midguts were carefully removed so that the food contents (containing potential substrates) of the guts were not disturbed. The guts were combined and homogenized in 2 ml of ice-cold 0.1 M sodium phosphate buffer (identical to the dissection buffer except that it contained in addition 1.25 mg leupeptin). One ml of homogenate was mixed with 9 ml of ice-cold 0.1 M sodium phosphate buffer (pH=7.8), and 1 ml of this mixture was pipetted into each of six 2-ml centrifuge tubes on ice, and the tubes were capped. The remaining 1 ml of homogenate was mixed with 9 ml 0.1 M sodium phosphate buffer (pH=7.8) containing an NADPH-regenerating system (10 mM NADP, 100 mM glucose-6-phosphate, and 0.5 units per ml of glucose-6-phosphate dehydrogenase), and 1 ml of this mixture was pipetted into each of six 2-ml centrifuge tubes on ice, and the tubes were capped. To three of the tubes for each reaction type, 300 μl of ethyl acetate were added, and the tubes were subsequently capped and vortexed. All of the tubes were placed at 30°C for 30 min, after which the tubes were returned to ice. Three hundred μl of ethyl acetate were added to the tubes not already containing ethyl acetate and these tubes were then recapped and vortexed. All of the tubes then were centrifuged at maximum speed for 2 min to ensure complete separation of organic and aqueous layers. After separation, 200 μl of organic layer were transferred to gas chromatography vials for analysis by gas chromatography–mass spectrometry (GC-MS). One microliter of each extract was analyzed by GC-MS (Hewlett Packard, 6890 GC attached to an HP 5972A Mass Selective Detector, Palo Alto, CA, USA) employing an Alltech (Deerfield, IL, USA) AT-5, 30 m x 0.25 mm column. The samples were run in splitless mode with an inlet temperature of 250°C and helium as the carrier. The initial oven temperature of 60°C was increased at 10°C/min until a final temperature of 300°C for 5 min. The mass spectrometer was set to scan from 50 to 550 m/z. Peaks were identified by matching to the NIST08 spectral library and by comparison with standards, if available. Amounts were quantified based on total ion chromatograms.

Confirmation of Carboxylesterase Activity The appearance of octanol in the metabolic profiling assay implicated esterase activity as the basis for metabolism of two aliphatic esters, octyl acetate and octyl butyrate, found in parsnip extracts. Some degree of spontaneous degradation of aliphatic esters in aqueous solutions is possible, however, which would also result in formation of octanol and an acid. Thus, to confirm that the disappearance of the aliphatic esters was due to webworm esterase activity, we included two additional treatments: guts filled with undigested food from *P. sativa* and guts cleaned of all contents of exogenous origin. We tested both groups to determine if plant-derived esterases from undigested gut materials might contribute to ester metabolism in the assay.

A total of 60 ultimate instar parsnip webworms were collected from a population in east Urbana, Champaign County, IL (coordinates 40.121173, −88.143400). The caterpillars were separated into 6 groups of 10 individuals. Midguts of caterpillars from each group were pooled for the enzyme assay. For three of the 6 groups, we cleaned the guts and removed all of the undigested plant material (Clean Gut treatment) and in the remaining three groups guts containing undigested food were assayed (Filled Gut treatment). Thus, each treatment was replicated three times. Caterpillars in each of the six groups were dissected in ice-cold dissection buffer [0.1 M sodium phosphate (pH 7.8), 0.25 M sucrose, 1% w/v polyvinylpyrrolidone, 1.1 mM ethylenediaminetetraacetic acid (EDTA), 2 mM phenylmethylsulfonyl fluoride (Crankshaw et al., 1979)] under a dissecting microscope, and midguts were collected. For the Clean Gut group, undigested food was carefully removed in ice-cold dissection buffer, and the guts were rinsed in fresh buffer. For the Filled Gut group, midguts were carefully pulled out so that the gut contents were not disturbed until homogenization.

Midguts from each group were pooled and homogenized in 5 ml of ice-cold grinding phosphate buffer [0.1 M sodium phosphate (pH 7.8), 0.25 M sucrose, 1% w/v polyvinylpyrrolidone, 1.1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM phenylmethylsulfonyl fluoride, and 5 ul/L leupeptin to inhibit proteinase activity]. The homogenate was diluted with 5 ml of ice-cold grinding phosphate buffer. One ml of homogenate was pipetted into each of six 2-ml centrifuge tubes per group. Two reactions and two controls were set up for each of the three substrates: reaction at time 0 (Rt0), reaction at time 30 min (Rt30), control at time 0 (Ct0), and control at time 30 min (Ct30). Reaction tubes contained gut homogenate and substrate whereas control tubes contained only cold grinding buffer and substrate. To stop the reaction, 300 μl of ethyl acetate were added to Rt0 and Ct0 tubes prior to incubation, after which the tubes were vortexed. To all reactions, 20 μl of 1% pure ester substrates–hexyl butyrate (SAFC, Lenexa, KS, USA), octyl acetate (ICN Pharmaceuticals Inc., Costa Mesa, CA, USA) and octyl butyrate (ICN Pharmaceuticals Inc., Costa Mesa, Inc.) were added.

All tubes then were incubated at 30°C for 30 min, after which the tubes were returned to ice. We added 300 μl of ethyl acetate to the Rt30 and Ct30 tubes and vortexed them.

All of the tubes were centrifuged at maximum speed for 2 min to ensure complete separation of organic and aqueous layers. The rationale behind running three separate controls (Rt0, Ct0, and Ct30) was to adjust for the spontaneous degradation of octyl butyrate and octyl acetate to octanol, and of hexyl butyrate to hexanol.

One microliter of the organic phase of each reaction was analyzed with a gas chromatograph—mass spectrometer (Shimadzu QP2010 Plus, SHRXI-5MS capillary column, 30 m×0.25 mm×0.25 μ) in splitless mode for 1.5 min with and inlet temperature of 250°C and helium as the carrier. The initial oven temperature of 50°C after 50 sec was increased at 10°C/min until a final temperature of 250°C for 5 min. The mass spectrometer was set to scan from 40 to 300 m/z. Peaks were identified by matching to the NIST08 spectral library. Amounts were quantified based on total ion chromatograms and compared to substrate and product standard (hexanol, octanol) curves to obtain absolute values.

Statistical Analysis In the metabolite profiling assay, one-way analysis of variance (ANOVA) was used to compare substrate levels between treatments; the buffer-only treatment compared substrate concentration before and after incubation with gut homogenate, and the NADPH treatment compared substrate concentrations between the buffer-only treatment (post incubation) and the incubations with NADPH present.

In the analysis to confirm esterase activity, substrate levels for all controls and reactions (Ct0, Ct30, Rt0, Rt30) were compared to each other to determine whether controls differed from each other and from the enzyme reaction. The comparisons were necessary to confirm that substrate disappearance was a result of the action of midgut esterases rather than spontaneous decomposition. Analysis of variance was used to compare differences in substrate disappearance when controlled for degradation (Ct30-Rt30), absorption by buffer (Rt0-Rt30), and combined degradation and buffer absorption [(Rt0-Rt30) - (Ct30-Ct0)]. Substrate disappearance was also compared between substrates (octyl acetate, octyl butyrate, and hexyl butyrate) and Clean Gut vs. Filled Gut treatments. Products of esterase metabolism (octanol and hexanol) also were compared between substrates and between the Clean Gut and Filled Gut treatments.

Results

Metabolic Profiling Experiment In the insect gut contents, we identified two monoterpenes(*cis*- and *trans*-ocimene), two sesquiterpenes(*β*-E-bergamotene and *trans*-*β*-farnesene), an apparent metabolite (octanol), several aliphatic esters (hexyl butyrate, octyl acetate, and octyl butyrate), several furanocoumarins (xanthotoxin, bergapten, and isopimpinellin), coumarin (osthol), and myristicin and palmitolactone (Fig. 1). All these compounds are also present in female wild parsnip flowers and developing fruits. The furanocoumarins, myristicin, osthol, and palmitolactone were metabolized in the presence of NADPH (significant difference between substrate concentration before and after incubation for all 6 compounds, $P<0.01$) but not in the buffer-only treatment, implicating cytochrome P450 involvement. Of the remaining substrates, all but *β*-E-bergamotene (buffer $P=0.894$, NADPH $P=0.341$) exhibited a significant decline after incubation in buffer with gut homogenate ($P<0.01$), whether NADPH was present or not. After further investigation of disappearance rates of pure compounds over time (data not shown), we concluded that disappearance of the terpenes cannot unambiguously be attributed to metabolism, and consequently they cannot be definitively designated as substrates.

Confirmation of Esterase Activity Substrate levels for Ct0 and Ct30 were different from each other (Fig. 2, $F=11.95$, $P<0.001$), suggesting that there is some amount of autodegradation, which must be taken into account in establishing whether insect enzymes are responsible for substrate disappearance. To account for substrate degradation, enzyme metabolism was calculated as [(Rt0-Rt30) - (Ct30-Ct0)]. Esterase activity in webworm midguts was confirmed with strong product (octanol and hexanol) peaks in the gut homogenate reaction tube (Rt30) as well as a significant difference between substrate disappearance in the reaction

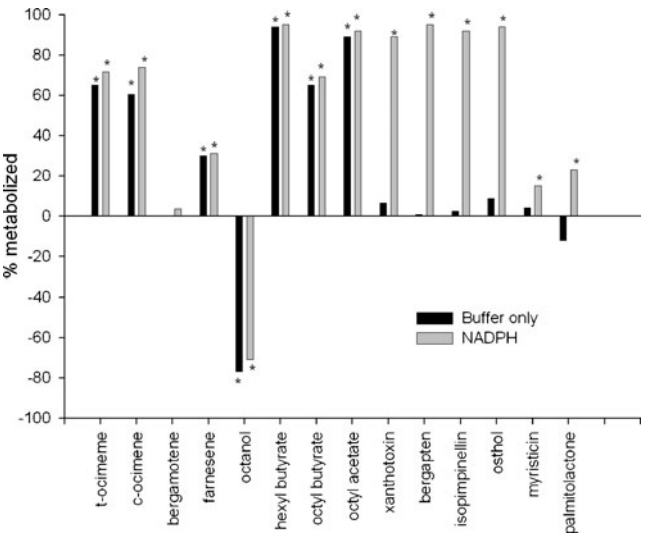

Fig. 1 Mean percentage of each of several host plant substrates metabolized by parsnip webworms (Rt0-Rt30/Rt0). Significant changes ($P<0.05$, one-way ANOVA) in substrate levels in comparisons between Rt0 and Rt30 are denoted by asterisks; the buffer-only treatment compares substrate concentration before and after incubation with gut homogenate and the NADPH treatment compares substrate concentrations between the buffer-only treatment (post incubation) and the incubations with NADPH present. Note that octanol increased during incubation, indicating that it is a product of metabolism

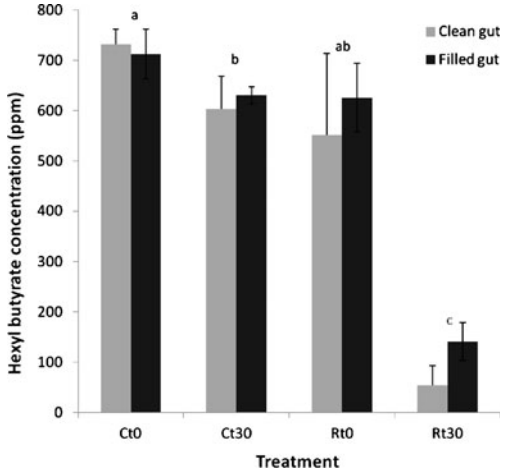

Fig. 2 Comparison of substrate amounts in different control (C, buffer only) and reaction groups (R, buffer and gut tissue) before and after incubation for the hexyl butyrate *in vitro* assay. Two-way analysis of variance revealed an overall effect of treatment; a *post-hoc* Tukey HSD test showed that filled and unfilled gut contents differ at Ct0 (**a**), at Ct30 (**b**) and at Rt30 (**c**), $P<0.05$). Results were similar for all three aliphatic esters

tubes (Rt0-Rt30) and autodegradation in the controls (Ct0-Ct30). Comparisons between cleaned and filled guts indicate that both substrate disappearance (Fig. 3, $F=18.54$, $P<0.001$) and product formation (Fig. 3, $F=23.12$, $P<0.001$) are higher in cleaned guts. Although we were concerned initially that plant esterases that remained active in the gut after ingestion might contribute to enzyme-mediated metabolism, we found that metabolic activity was actually higher in cleaned guts, raising the possibility that some plant constituents in gut contents could inhibit insect esterase activity to some extent.

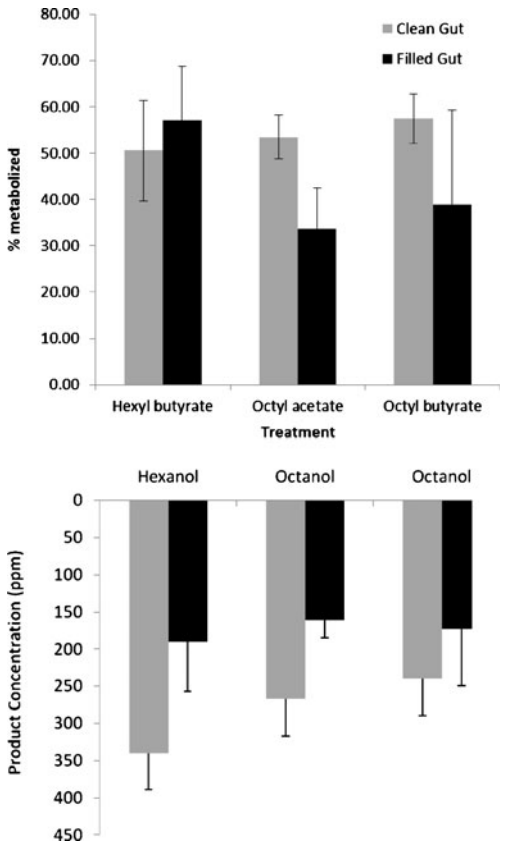

Fig. 3 Metabolism of aliphatic esters *in vitro* with clean and filled webworm midguts. Percentage substrate metabolism calculated as (Rt30-Rt0)-(Ct30-Ct0)/Ct0 (*top*) and concentration of product (*bottom*)

Discussion

Esters are common components of floral volatiles and can function both in the attraction of mutualistic pollinators and in the defense against antagonistic herbivores. Despite the importance of esters in mediating plant-insect interactions, little is known about insect metabolism of these compounds. As a specialist on the floral tissues of wild parsnip and related species, the parsnip webworm routinely encounters octyl esters in its diet; octyl esters are major constituents of the essential oil of *P. sativa* (Carroll et al., 2000). While octyl acetate is a behavioral attractant for foraging larvae, the closely related octyl butyrate is both repellent and toxic to larvae (Carroll and Berenbaum, 2001). Concentrations of these two esters vary developmentally and geographically, but octyl acetate can reach concentrations up to 5.5 μg/mg dry weight and octyl butyrate up to 14.22 μg/mg dry weight in seeds (Carroll et al., 2000).

How *D. pastinacella* tolerates these esters in its diet has not been explored; in fact, despite the widespread distribution of aliphatic esters in plant tissues ingested by insects, their degradation by midgut esterases has not been documented previously. The disappearance of the three aliphatic esters found in abundance in parsnip floral tissues and the production of the corresponding alcohols is consistent with detoxification by carboxylesterases. Esterase-mediated metabolism of these compounds in parsnip webworms may reflect the ecological significance of these esters in mediating webworm florivory. Octyl butyrate, generally the most abundant ester in the plant reproductive structures consumed by webworms, functions as a deterrent to larval feeding, whereas octyl acetate acts as a larval attractant (Carroll and Berenbaum, 2001). Consistent with these behavioral effects, wild parsnips in New Zealand, which had been free of infestation by the parsnip webworm for over 100 years until its accidental introduction in 2004, produced larger amounts of octyl acetate and smaller amounts of octyl butyrate than their North American counterparts. These differences may reflect longstanding selection in North America that has reduced apparency to and suitability of floral tissues for parsnip webworms (Zangerl et al., 2008). Webworm pupal mass in New Zealand is positively correlated with octyl acetate and negatively

correlated with octyl butyrate levels in seeds, suggesting that New Zealand parsnips may, after a sufficiently long period of selection by webworms, adapt to resemble North American parsnips more closely in terms of octyl ester production. The 19-fold difference in the rate of disappearance of octyl butyrate and octyl acetate is suggestive of considerable substrate specificity of the esterase or esterases involved in detoxification of these compounds.

To date, the overwhelming majority of studies of esterase contributions to xenobiotic metabolism have focused on their role in resistance to organophosphate (Srigiriraju et al., 2009; Rose et al., 2011) and other insecticides (Abd El-Latif and Subrahmanyam, 2010; Farnsworth et al., 2010; Feng et al., 2011). Contributions of esterases to host plant utilization by insect herbivores have been largely overlooked. Their function in adaptations of papilionid swallowtails to their hostplants was suggested over twenty years ago, when Lindroth (1989a) demonstrated differential esterase activity in *P. glaucus* and *P. canadensis* (considered subspecies at the time), with *P. canadensis* displaying higher levels of esterase activity against phenolic glycosides typical of *P. canadensis* host plants. Lindroth (1989b) also showed that the esterases responsible for phenolic glycoside metabolism may be somewhat specialized in that they are not highly active against organophosphate insecticides. Other studies documenting metabolism of phytochemicals by carboxylesterases did not appear until Durand et al. (2010) demonstrated that the esterase SICXE10 in both larval and adult antennae of *Spodoptera littoralis* hydrolyzes the green leaf volatile (Z)-3-hexenyl acetate (*Z*3-6:Ac) (albeit with low affinity); the ecological function of *Z*3-6:Ac, in terms of host-finding and assessment, is as yet undetermined. Our findings with the parsnip webworm suggest that esterases may in fact play an important role in larval lepidopteran adaptation to hostplant defensive chemistry.

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Influence of Phenological Stage on Swainsonine and Endophyte Concentrations in *Oxytropis sericea*

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Abstract Locoweeds are defined as *Astragalus* and *Oxytropis* species that cause intoxication due to the alkaloid swainsonine. Swainsonine concentrations in *Oxytropis sericea* were influenced by location, plant part, and the developmental stage of the plant. Concentrations followed similar trends at each location, generally increasing over the growing season in above-ground parts until the plant reaches maturity with no change in concentration in the crowns. At the onset of senescence, swainsonine decreased in floral parts to less than half of the peak concentration. Similar to swainsonine concentrations, endophyte amounts were influenced by location, plant part, and the developmental stage of the plant. Likewise, endophyte amounts generally increased over the growing season in above ground parts and remained static in the crowns at all four locations. Swainsonine in *Oxytropis sericea* was positively associated with the endophyte *Undifilum*, which is responsible for swainsonine biosynthesis.

Keywords Locoweed · *Astragalus* · *Oxytropis* · Swainsonine · *Undifilum* · Endophyte · Phenology

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Introduction

Locoweeds (*Astragalus* and *Oxytropis* spp.), members of the Legume family, are common toxic plants in the western United States that frequently poison livestock (Kingsbury, 1964; Cook et al., 2009a). Economic impact, such as death and abortion, reaches several million dollars per year (James and Nielsen, 1988). Locoweed intoxication occurs worldwide; *Astragalus* and *Oxytropis* species that contain swainsonine have poisoned animals in South America and Asia (Molyneux and Gomez-Sosa, 1991; Cao et al., 1992; Molyneux et al., 1994). Additionally, other plants contain swainsonine and poison animals, including *Swainsona canescens* in Australia (Colegate et al., 1979) and some *Ipomoea*, *Sida*, and *Turbina* species in South America and Africa (Molyneux et al., 1995; Colodel et al., 2002; Dantas et al., 2007).

Swainsonine, a trihydroxy indolizidine alkaloid, is the primary toxin in locoweeds (Molyneux and James, 1982). The vertically transmitted fungal endophyte *Undifilum* found in locoweeds (Oldrup et al., 2010; Ralphs et al., 2011), previously described as *Embellisia* species, is responsible for the synthesis of swainsonine (Braun et al., 2003; Wang et al., 2006; Pryor et al., 2009). Swainsonine concentrations vary greatly in locoweeds; for example, swainsonine may or may not be detected within varieties, populations, and/or individuals within the same population of *Astragalus* and *Oxytropis* species (Gardner et al., 2001; Ralphs et al., 2008; Cook et al., 2009b, 2011). Differences in the amount of *Undifilum* have been associated with the highly variable swainsonine concentrations in *Oxytropis* and *Astragalus* (Cook et al., 2009b, 2011). For example, *Astragalus lentiginosus* and *A. mollissimus* plants with swainsonine concentrations greater than 0.1%

had greater than 20 times the amount of *Undifilum* than comparable plants of the same species with swainsonine concentrations less than 0.01% (Cook et al., 2011).

Swainsonine and endophyte amounts differ among plant parts in *O. sericea* (Cook et al., 2009b). Above ground parts (leaves, scapes, flowers/pods) had greater than 10 times the amount of swainsonine than below ground parts (roots, crowns). Endophyte amounts also differed among plant parts. Generally, above ground parts (leaves, scapes, flowers/pods) had greater amounts than below ground parts (roots, crowns) with the exception of the crown, which may serve as an endophyte reservoir for the following years' growth (Cook et al., 2009b). These previously reported results were based upon a single phenological stage and location and provided no indication whether or not swainsonine and endophyte amounts change in plants parts as a function of plant development or if they differ between geographical locations. To better understand how amounts are influenced by plant growth and development in *O. sericea*, swainsonine and endophyte concentrations were measured in different plant parts, at five developmental stages, and at four different geographic locations in the western United States.

Methods and Materials

Plant Materials *Oxytropis sericea* samples were collected at four locations (Hanksville, UT; Cuchara, CO; Hartsel, CO; and Park Valley, UT) in 2010. A distribution map of *O. sericea* and the 4 collections sites is shown (Fig. 1). Elevations at the 4 sites were 9,609, 9,957, 8,985, and 7,778 feet, respectively. Collection sites and dates of collection are listed in Table 1. Plants (15-25) were sampled destructively approximately every month at each location to obtain distinct phenological stages associated with plant growth and development [vegetative (stage 1), early flower (stage 2), late flower/early pod (stage 3), full pod/mature seed (stage 4), and senescing plants (stage 5)]. Collections at Hartsel, CO differed from the other locations: first, approximately 20% of the plants initiated flowering in stage 1, and second, all flowers and developing pods aborted between stage 3 and 4 resulting in dried scapes that were used for time points 4 and 5. Plants were divided into 3 parts: crown (persistent base of the herbaceous perennials, non-photosynthetic woody tissue extending from ground level to tap root), leaves (odd-pinnate with leaflets being opposite), and floral parts (scapes, flowers, and/or pods). All parts were sampled from each plant. Immediately after collection, plant material was bagged and frozen on dry ice. Upon return to the laboratory, plants were freeze-dried and ground through a 2 mm screen in a Wiley mill. Swainsonine and DNA were extracted for further analyses.

Swainsonine Analysis Swainsonine detection and concentration was measured using a modification of a previously published procedure (Gardner and Cook, 2011). Dried plant material (50 mg) was placed in a 2 ml screw-cap microcentrifuge tube. Ground plant material was extracted in 1.5 ml of 2% acetic acid for 18 h with agitation. After extraction, samples were centrifuged and 0.05 ml of extract was added to 0.95 ml of 20 mM ammonium acetate in a 1 ml auto-sampler vial. Samples were analyzed by LC-MS as previously described (Gardner et al., 2001). Detection limit of swainsonine was 0.001% of dry weight using this extraction procedure.

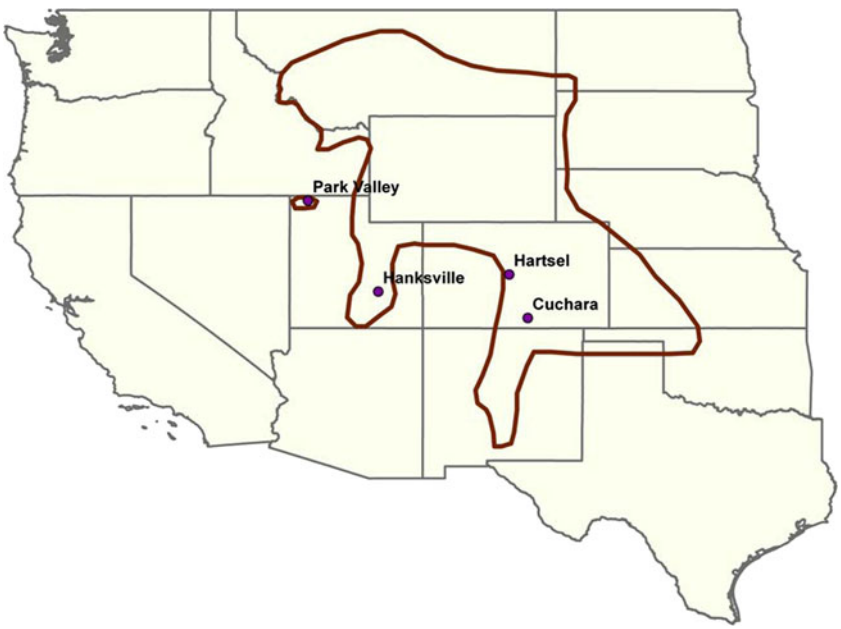

Fig. 1 Distribution map of *Oxytropis sericea* showing the four collections sites. Hanksville, UT; Cuchara, CO; Hartsel, CO; and Park Valley, UT

Table 1 Collections sites, phenological stage, and date of collection of *Oxytropis sericea* populations

Sites	Location (GPS coordinates)	Phenological Stage / Collection date				
		Vegetative	Early flower	Late flower Early pod	Full pod Mature seed	Senesced
Hanksville, UT	N 38°4′15.6″ W 110° 48′ 35.6″	5/19/2010	6/15/2010	7/19/2010	8/18/2010	10/04/2010
Cuchara, CO	N 37°19′15.7″ W 105° 04′23.8″	6/04/2010	6/26/2010	7/29/2010	9/01/2010	10/18/2010
Hartsel, CO	N 38°57′58.3″ W 105° 43′ 32.2″	6/04/2010	6/26/2010	7/29/2010	9/01/2010	10/18/2010
Park Valley, UT	N 41°55.910′ W 113° 28.174′	5/17/2010	6/16/2010	7/14/2010	8/16/2010	09/15/2010

DNA Extraction DNA was extracted from freeze-dried, ground plant material (~20 mg) using the DNEasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA). Extractions were performed according to the manufacturer's instructions. DNA was quantified with the ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

PCR Primers The PCR primers used have successfully detected the presence of the fungal endophyte, *U. oxytropis* in *Oxytropis* and *Astragalus* species (Ralphs et al., 2008; Cook et al., 2009a, b, 2011). Primers used were ITS 5 (5′ GGA AGT AAA AGT CGT AAC AAG G 3′) (White et al., 1990) and OR1a (5′ GTC AAA AGT TGA AAA TGT GGC TTG G 3′), which amplify the internal transcribed spacer (ITS) region. Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA).

Endophyte Quantitation A standard curve (30, 10, 3, 1, 0.3, 0.1, 0.03, 0.01 ng fungal DNA) was prepared from DNA extracted from a pure culture of the endophytic fungus for endophyte quantitation (Cook et al., 2009c). Each analysis included three replicate reactions (25 μl) for each DNA quantity. Each analysis also included a non-template control reaction, in which water was substituted for the DNA to confirm that reagents were free from contaminating template DNA. The equation derived from the standard curve was $y = -3.54x + 17.9$, R^2=0.996.

Plant samples were analyzed in three replicate 25 μl reactions containing 50 ng (5 μl of a 10 ng / μl stock) of total DNA. Each reaction contained 12.5 μl of the QuantiFast SYBR Green PCR Kit master mix (Qiagen Inc., Valencia, CA, USA) and 500 nM each of the forward and reverse primer. Endophyte content (per 50 ng total DNA) was estimated from the endophyte standard curve. Endophyte amounts were expressed as picograms/nanogram total DNA.

Amplification and detection of fluorescence were performed using a Bio-Rad CHROMO4 quantitative PCR detector (Bio-Rad Laboratories Inc., Hercules, CA, USA). Thermal cycling conditions comprised an initial denaturation step for 7 min at 95°C, followed by 40 cycles of 15 sec at 95°C, 30 sec at 58°C, 40 sec at 72°C, and a plate read at the end of each cycle. This was followed by a melting profile, to determine the purity of the reaction products, where the temperature was raised from 55°C to 90°C in 0.2°C increments, held for 2 sec at each temperature, and a plate read at each temperature.

Data Analysis Plants from each location and time point were first separated into two groups by using externally determined criteria based upon previous observations (Cook et al., 2009b, 2011): chemotype 1 plants that contained swainsonine concentrations greater than 0.01% in the leaves or flowers; or chemotype 2 plants that contained swainsonine concentrations less than 0.01% in the leaves or flowers. Up to a maximum of 12 chemotype 1 plants (range of 6–12 with a mean of 11.3 for the four locations), depending on the number available, were selected at each location at each sample period by using a stratified selection based upon swainsonine concentrations. All plant parts from these plants were analyzed subsequently for swainsonine and endophyte amounts.

Endophyte amounts (pg/ng of total DNA) were transformed to natural log for statistical comparisons; untransformed values are shown in graphs and tables unless otherwise noted. Swainsonine concentrations and log-transformed endophyte concentrations in each plant part from five stages were examined using a general linear model of SAS (PROC GLM) (SAS Institute, Cary, NC, USA). Swainsonine concentrations and log transformed endophyte concentrations were compared by stages, locations, and parts using the GLM procedure with least squares means for unbalanced sample sizes. Preplanned comparisons were performed using the PDIFF procedure in SAS. Swainsonine (Supplemental Table 1) and endophyte amounts (Supplemental Table 2) as well as statistical comparisons between parts and time points at each location are included as supplemental tables.

Regression analysis was performed comparing swainsonine concentrations (%) and endophyte amounts [ln(pg/ng)] using Microsoft Excel. An exponential regression line was determined to best fit the data. For regression analysis, the following phenological stages were used: crowns, stages 1–

5; leaves, stages 1–4; and floral stages 2–4 (with the exception of Hartsel, CO; floral stages 1–3). Stage 5 was not included for aboveground parts as the plants reached full maturity at stage 4 and were beginning to senesce at stage 5.

Results

Swainsonine The percentage of surveyed plants belonging to chemotype 1 from each population was 77% at Cuchara, CO, 40% at Hanksville, UT; 66% at Hartsel, CO; and 89% at Park Valley, UT. Concentrations differed by plant part x phenological stage x location ($P<0.001$). The main effects as well as two way interactions were significant ($P<0.001$). Swainsonine was detected in all plant parts but concentrations were greater in above ground than below ground parts ($P<0.001$) as previously reported (Cook et al., 2009b). Swainsonine concentrations differed among above ground parts as reported by Cook et al. (2009b) with differences being related to phenological stage (Fig. 2). For example, at Cuchara, CO, swainsonine concentration in leaves at stage 2 (0.17%) had greater concentrations than floral parts at stage 2 (0.06%), while at stage 4, floral parts (0.36%) had greater concentrations than leaves (0.26%) (Fig. 2b).

Swainsonine concentrations in the different plant parts showed similar trends over time, although the absolute amounts may vary among locations (Fig. 2). Concentrations in the crowns were generally constant over the growing sea-

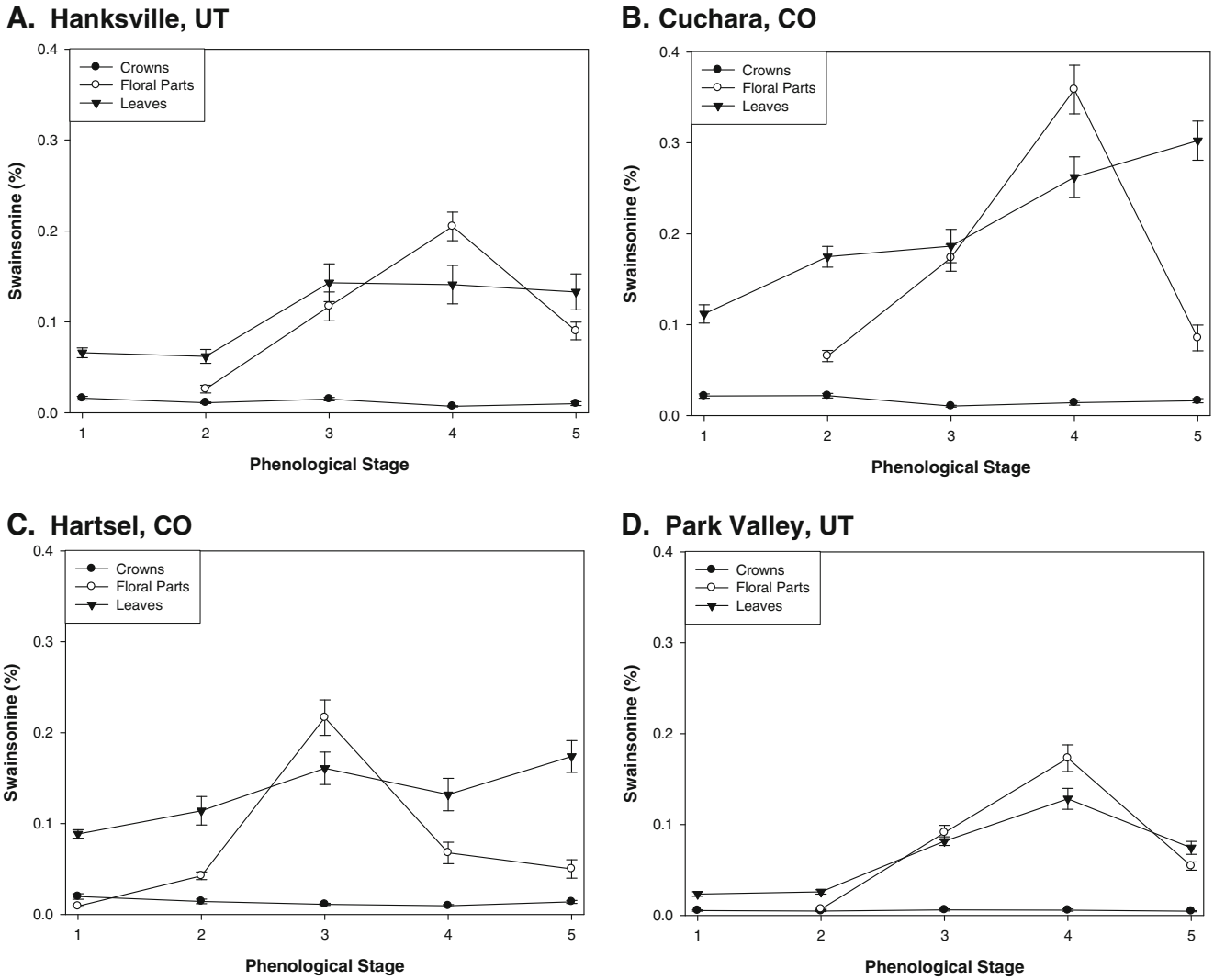

Fig. 2 Swainsonine concentrations (%) in different plant parts of *Oxytropis sericea* as a function of phenological stage. Mean swainsonine concentrations±the standard error at the different phenological stages (stage 1, vegetative; stage 2, early flower; stage 3, late flower/early pod; stage 4 full pod/mature seed; stage 5, senesced) at the four collection sites. **A** Hanksville, UT; **B** Cuchara, CO; **C** Hartsel, CO; and **D** Park Valley, UT

son at each location (Fig. 2); for example, concentrations in the crowns from plants at Cuchara, CO ranged from a minimum of 0.01% at stage 3 to a maximum of 0.02% at stage 2 (Fig. 2b). Unlike the crowns, swainsonine concentrations in leaves increased over the year at each location reaching the maximum at stage 4 or 5 (Fig. 2). For example, concentrations in leaves from plants sampled at Cuchara, CO were 0.11% at stage 1 and increased over the growing season to a maximum of 0.30% at stage 5 (Fig. 2b). As in the leaves, swainsonine concentrations in floral parts also increased over the growing season at each location reaching the maximum at stage 4 followed by a subsequent decrease at stage 5 (Fig. 2). Illustrating this trend, concentration in floral parts from plants collected at Cuchara, CO were 0.06% at stage 2, increased to a maximum of 0.36% at stage 4, and subsequently decreased to 0.08% at stage 5 (Fig. 2b).

Similar trends in swainsonine concentrations in the different parts over time were observed with two exceptions (Fig. 2). First, at Hartsel, concentrations in floral parts decreased from 0.22% at stage 3 to 0.07% at stage 4 (Fig. 2c), while the other locations continued to increase at stage 4 and decrease at stage 5. Second, at Park Valley, UT, concentrations in leaves decreased from 0.13% at stage 4 to 0.07% at stage 5 (Fig. 2d), while at the other locations concentrations increased or stayed the same.

Swainsonine concentrations differed between locations as a function of part and time (Fig. 2). For consistency, comparisons are made only between the same plant parts at the same time point at different locations. For example at Cuchara, CO at time point 3, leaves (0.19%) and flowers (0.17%) (Fig. 2b) contained greater amounts of swainsonine than leaves (0.08%) and flowers (0.09%) at Park Valley, UT at time point 3 ($P<0.001$) (Fig. 2d). In general, leaves and flowers had greater amounts throughout the entire year at Cuchara, CO compared to Park Valley, UT when comparing the same time point (Fig. 2). Concentrations at Hanksville, UT usually were intermediate to Cuchara, CO and Park Valley, UT (Fig. 2) when comparing the same plant part and time point. Concentrations at Hartsel, CO were difficult to compare to the other three locations because the flowers and early pods aborted between stage 3 and 4; however, based on early time points, it appears as though concentrations at Hartsel, CO were intermediate or more like Cuchara, CO.

Endophyte Endophyte concentrations showed a trend towards differing by plant part x phenological stage x location ($P=0.11$). The main effects as well as two way interactions also were significant ($P<0.001$). Endophyte amounts differed between above ground parts with differences being related to the phenological stage (Fig. 3). For example, at Cuchara, CO, stage 2 leaves (2.4 pg/ng) had similar amounts of endophyte to floral parts (2.3 pg/ng), while at stage 4, floral parts (11.6 pg/ng) had greater amounts than leaves (6.1 pg/ng) (Fig. 3b).

Endophyte amounts in different plant parts showed similar trends over time at each location although the absolute amount of endophyte varied among locations (Fig. 3). Amounts in the crowns differed between time points in some instances but no characteristic pattern was observed over time at the four locations (Fig. 3). For example, amounts in the crowns at Cuchara, CO ranged from a minimum of 0.7 pg/ng at stage 1 to a maximum of 2.2 pg/ng at stage 3 (Fig. 3b), while other locations showed different trends. Unlike the crowns, endophyte amounts in leaves generally increased over the year with a minimum amount in stage 1 and reaching a maximum amount at stage 4 or 5 (Fig. 3). Illustrating this trend, amounts in leaves from plants samples at Cuchara, CO increased from a minimum of 1.0 pg/ng at stage 1 to a maximum of 6.1 pg/ng at stage 4 (Fig. 3b). As in the leaves, amounts in the floral parts also increased over the year reaching a maximum at stage 5 (Fig. 3). For example, amounts in leaves from plants collected at Cuchara, CO increased from a minimum of 1.0 pg/ng at stage 2 to a maximum of 16.3 pg/ng at stage 5 (Fig. 3b).

Endophyte amounts differed between locations as a function of part and time (Fig. 3). As with swainsonine concentrations, comparisons for endophytes were made only between the same plant parts at the same time point at different locations. As a case in point, at Park Valley, UT, at time point 4, leaves (20.6 pg/ng) and floral parts (30.7 pg/ng) (Fig. 3d) contained greater amounts of endophyte than leaves (6.0 pg/ng) and floral parts (11.6 pg/ng) at Cuchara, CO at time point 4 ($P<0.001$) (Fig. 3b). In general, time points 1, 2, and 3 had similar amounts of endophyte among plant parts at the different locations, while greater differences were observed in amounts among parts at the different locations as plants matured at time points 4 and 5 (Fig. 3).

Swainsonine/Endophyte Relationship To further explore the relationship between swainsonine and endophyte, regression analysis was performed comparing swainsonine and endophyte amounts. Positive relationships ($R^2 \geq 0.47$) between endophyte amounts and swainsonine concentrations were found in leaves and floral parts at all four locations (Table 2). Figure 4 shows the regression analysis that compared swainsonine and endophyte amounts of the crowns, leaves, and floral parts at Cuchara, CO. Endophyte amounts could explain 47–62% of the total variation in swainsonine concentration in leaves, or 59–85% of the total variation in swainsonine concentration in floral parts, with differences being dependent upon location (Table 2). Unlike leaves and floral parts, endophyte amounts in the crowns explained little or none of the variation in swainsonine concentration observed in the crowns.

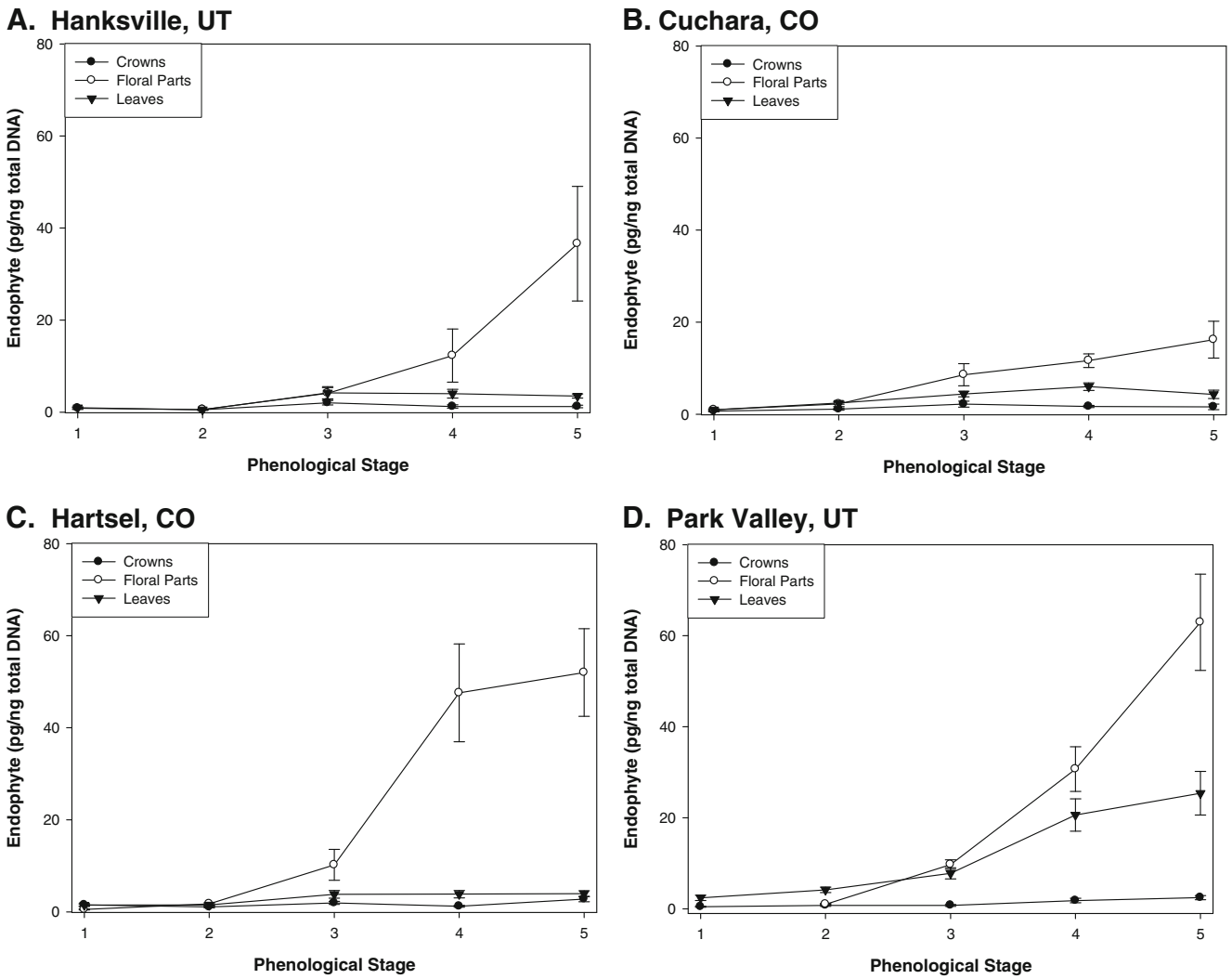

Fig. 3 Endophyte amounts (pg/ng) in different plant parts of *Oxytropis sericea* as a function of phenological stage. Mean endophyte amounts± the standard error at the different phenological stages (stage 1, vegetative; stage 2, early flower; stage 3, late flower/early pod; stage 4 full pod/mature seed; stage 5, senesced) at the four collection sites. **A** Hanksville, UT; **B** Cuchara, CO; **C** Hartsel, CO; and **D** Park Valley, UT

Discussion

By using methods previously developed to quantitate swainsonine and endophyte, both were investigated over the growing season representing 5 phenological stages in different plant parts at 4 locations of *O. sericea*. Swainsonine and endophyte amounts showed similar trends at the 4 locations surveyed although the absolute amounts differed among locations. The seasonal changes in swainsonine reported here are similar to those observed in lolines and ergovaline in endophyte-infected meadow fescue and tall fescue, respectively (Rottinghaus et al., 1991; Justus et al., 1997). We believe that changes in swainsonine and endophyte amounts are likely to occur on an annual basis, as similar observations have been made for *O. sericea* at Park Valley, UT in a previous year (data not shown). We speculate that similar trends may be observed in Astragalus locoweeds, although this merits further investigation.

Additionally, the results showed a positive relationship between swainsonine and endophyte amounts over the distinct phenological stages as the plant matures. Increases in

Table 2 Exponential regression analysis (R^2 values) comparing *Undifilum oxytropis* content (ln(pg/ng)) and swainsonine concentrations (%) at four collection sites in different plant parts

Location	Hanksville, UT	Cuchara, CO	Hartsel, CO	Park Valley, UT
Crowns	0.14	0	0.12	0.16
Leaves	0.47	0.62	0.48	0.55
Floral Parts	0.77	0.59	0.6	0.85

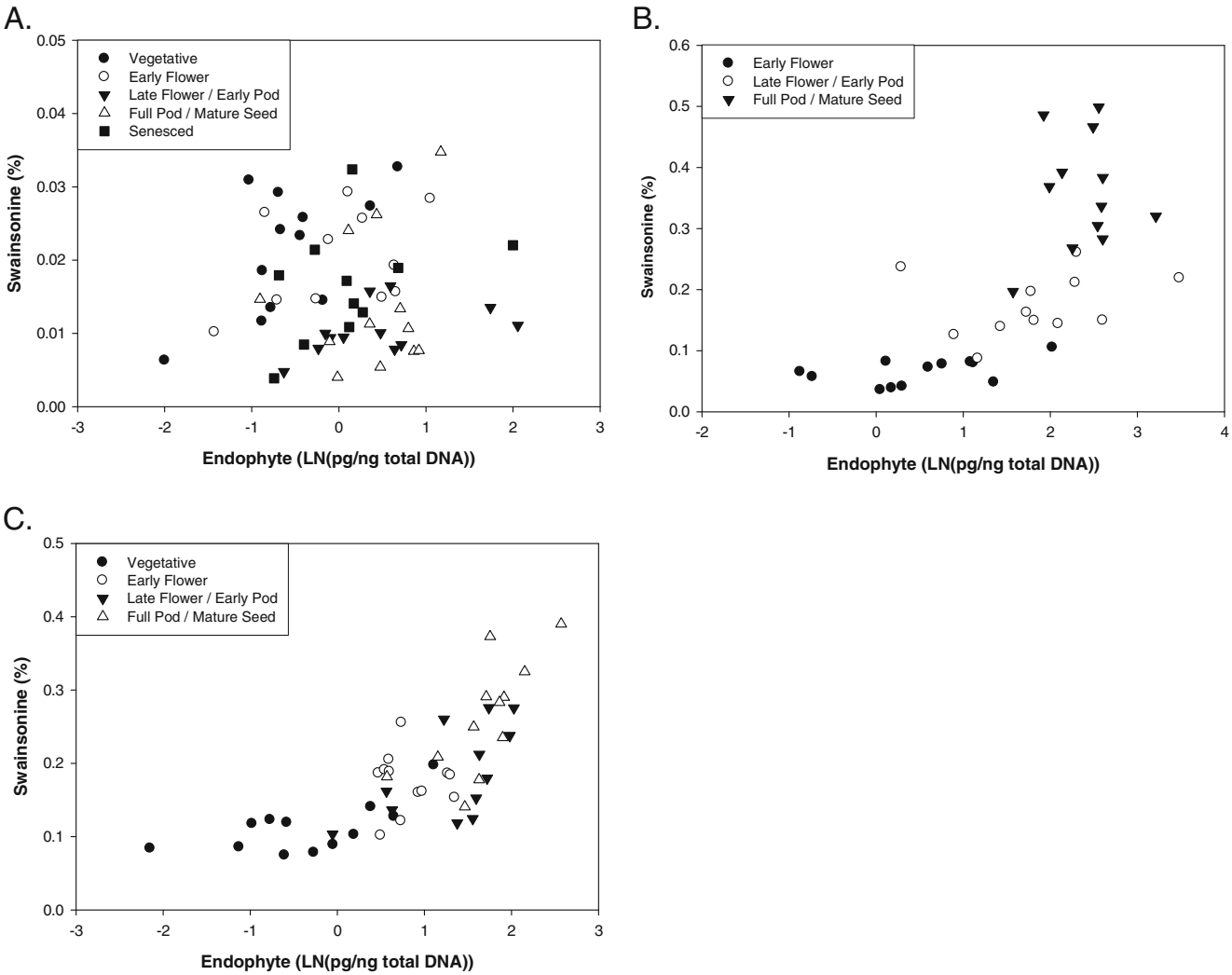

Fig. 4 Scatter plot showing relationship between swainsonine (%) and endophyte amounts (ln(pg/ng)) in different plant parts of *Oxytropis sericea* over the growing season at Cuchara, CO. **A** crowns; **B** floral parts; and **C** leaves

endophyte amounts are correlated with an increase in swainsonine concentrations in above ground parts. These results are consistent with observations made in regard to the *Neotyphodium* perennial rye grass symbiosis, in which the concentrations of some secondary compounds produced by the fungal endophyte, such as peramine and lolitrem B, are positively correlated with endophyte amounts, but not others, such as ergovaline (Young et al., 2005).

Previously, Cook et al. (2009b) reported results for swainsonine and endophyte amounts in *O. sericea* from Park Valley, UT from plants that were collected at a developmental stage similar to time point 3. The results reported here for time point 3 are similar, with the exception that endophyte amounts in the crowns were lower than previously reported. This may be due to how the crowns and leaves were sampled or to the subset of plants sampled. Nonetheless, endophyte amount in the crowns, although smaller than previously observed, may serve as a reservoir of the endophyte for subsequent growth in the following year, as has been proposed in locoweeds (Cook et al., 2009b, 2011) and for endophyte-containing grasses (Keogh et al., 1996).

Previous investigators have suggested that swainsonine is a potential mobile secondary compound that is transported in the phloem (Dreyer et al., 1985). Although not tested directly, the results from this experiment, as well as observations made by Cook et al. (2009b, 2011) do not support the idea that swainsonine is highly mobile within the plant. If it were, one would expect concentrations to be more uniform throughout the plant. The results reported here where we examined swainsonine concentrations changes in the different tissues over time are not consistent with the mobility hypothesis. For example, swainsonine concentrations found in newly developing floral parts are significantly

lower than those found in leaves at a comparable phenological stage. Additionally, the previous observation made by Cook et al. (2009b) concerning the very small amounts of swainsonine observed in roots and crowns suggests that swainsonine is not highly mobile.

The seasonal changes in swainsonine are similar to those observed in lolines and ergovaline in endophyte-infected meadow fescue and tall fescue, respectively (Rottinghaus et al., 1991; Justus et al., 1997). We hypothesize that this decrease at stage 5 is triggered by senescence. Other possible explanations may be that swainsonine is in flux throughout the growing season, but as the plant starts to senesce the rate of catabolism is greater than its biosynthesis due to the availability of photosynthate. Alternatively, swainsonine has a low C:N ratio, and thus, nitrogen may be remobilized for the plant to prepare for the next growing season. Finally, this decrease could be due simply to the loss of seeds from the floral parts from stage 4 to 5. This is not likely, however, as there was a large number of seeds still present in the pods when the plants were collected at stage 5. These ideas are speculative at this point, as the benefit or role swainsonine and/or the endophyte are providing, if any, to the plant still are not known.

The seasonal changes in swainsonine concentrations suggest that *O. sericea* poses the greatest toxic risk to livestock when the plants are at full pod/mature seed stage. However, toxicity also is influenced by the palatability and the availability of locoweeds relative to other available forage (Ralphs, 1987; Ralphs et al., 1993, 1994). Based upon grazing studies performed in New Mexico, Utah, and Colorado, cattle readily graze *O. sericea* during flower to early pod stage of growth, which correlates to phenological stage 3. Such studies have shown also that livestock will graze locoweeds earlier and later than this stage, but diet selection of livestock is highly dependent upon other available forage (Ralphs, 1987; Ralphs et al., 1993, 1994).

Two other observations of particular interest from the data presented here merit further investigation. First, differences in swainsonine concentrations between Park Valley, UT and Cuchara, CO need further study. Such differences may be due to genotype of the endophyte associated with each population, the genotype of the plants at each population, the environment and its effect on plant and endophyte, and/or a combination of these factors. Second, the decrease in swainsonine concentrations in floral parts and possibly in leaves remains to be investigated.

In summary, there were trends in swainsonine and endophyte amounts over the growing season that were similar at the four locations surveyed. Swainsonine concentrations peak as seeds mature with concentrations being the highest in the floral parts. Endophyte and swainsonine content are positively related.

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Stability of Plant Defensive Traits Among Populations in Two *Eucalyptus* Species Under Elevated Carbon Dioxide

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Abstract Plant secondary metabolites (PSMs) mediate a wide range of ecological interactions. Investigating the effect of environment on PSM production is important for our understanding of how plants will adapt to large scale environmental change, and the extended effects on communities and ecosystems. We explored the production of PSMs under elevated atmospheric carbon dioxide ($[CO_2]$) in the species rich, ecologically and commercially important genus *Eucalyptus*. Seedlings from multiple *Eucalyptus globulus* and *E. pauciflora* populations were grown in common glasshouse gardens under elevated or ambient $[CO_2]$. Variation in primary and secondary chemistry was determined as a function of genotype and treatment. There were clear population differences in PSM expression in each species. Elevated $[CO_2]$ did not affect concentrations of individual PSMs, total phenolics, condensed tannins or the total oil yield, and there was no population by $[CO_2]$ treatment interaction for any traits. Multivariate analysis revealed similar results with significant variation in concentrations of *E. pauciflora* oil components between populations. A $[CO_2]$ treatment effect was detected within populations but no interactions were found between elevated $[CO_2]$ and population. These eucalypt seedlings appear to be largely unresponsive to elevated $[CO_2]$, indicating stronger genetic than environmental (elevated $[CO_2]$) control of expression of PSMs.

Keywords Elevated CO_2 · *Eucalyptus* · Genetic variation · Plant secondary metabolites · PSM · Carbon dioxide · Terpene · Essential oil · Phenolic · Condensed tannin

Introduction

Plant secondary metabolites (PSMs) mediate a wide range of ecological interactions including herbivory (O'Reilly-Wapstra et al., 2004; Moore et al., 2005; Gershenzon and Dudareva, 2007). Variation in the environment can affect the expression of PSMs in many species (Fritz, 1999) due to interactions between genes and environment (GxE). Investigating how stable the expression of PSMs is across different environments (Andrew et al., 2010) is important for our understanding of the impact of large scale environmental changes on ecosystems (Whitham et al., 2006).

One large scale environmental change of current importance is the increase in atmospheric carbon dioxide concentration ($[CO_2]$) (IPCC, 2007). Increasing $[CO_2]$ is expected to affect a wide range of species and ecological processes (Hovenden and Williams, 2010; Lindroth, 2010) including plant growth, plant physiological processes, and biotic community composition (Long et al., 2004; Lindroth, 2010). Elevated $[CO_2]$ may alter plant chemical composition because CO_2 influences plant nutrient assimilation, carbon assimilation, the abundance of PSM biosynthetic precursors, and possibly signal transduction pathways that control gene expression and subsequent PSM synthesis (Lindroth, 2010). Elevated $[CO_2]$ may increase or decrease concentrations of foliar PSMs (Hovenden and Williams, 2010), altering plant interactions with other biota and modifying ecosystems.

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Eucalyptus is a species-rich tree genus in Australia (over 700 species) and is commonly the dominant species in forest stands (Brooker, 2002; Butcher et al., 2009). Eucalypt foliage contains a diversity of PSMs that vary qualitatively and quantitatively between species, and quantitatively within species (Li et al., 1995, 1996; Bignell et al., 1998; O'Reilly-Wapstra et al., 2010). The phenotypic expression of many of these compounds is under strong genetic control (Freeman et al., 2008) and affects many ecological interactions. For example, the terpene 1,8-cineole defends against herbivores (Wiggins et al., 2003), formylated phloroglucinol compounds (FPCs) are important anti-feedants to marsupial herbivores (Moore et al., 2004; Wiggins et al., 2006; Andrew et al., 2007; O'Reilly-Wapstra et al., 2010), and tannins affect browsing (Wallis et al., 2002). These compounds also affect ecosystem processes such as foliar flammability (Holmes, 2009; Steinbauer, 2010).

Despite the diversity and ecological role of PSMs in eucalypts, few studies have investigated the impact of elevated [CO_2] on PSMs in *Eucalyptus* species (Lawler et al., 1997; Gleadow et al., 1998). Both species-specific and intraspecific responses to elevated [CO_2] have been highlighted in other species (Bidart-Bouzat and Imeh-Nathaniel, 2008; Lindroth, 2010). To our knowledge, the effect of elevated [CO_2] on intra-specific variation in PSM concentrations within a *Eucalyptus* species has not yet been examined.

Here, we focused on PSMs in populations of two eucalypt species. *Eucalyptus globulus* Labill. is dominant in forest stands in south-eastern Australia and is the most widely planted hardwood species in temperate regions of the world (Eldridge et al., 1993). *Eucalyptus pauciflora* Sieber ex Sprengel. (subsp. *pauciflora*) is an important restoration species used in plantings throughout Tasmania and mainland Australia (Close et al., 2005, 2010). These species are from different *Eucalyptus* subgenera (*E. globulus* from subgenus *Symphyomyrtus*, *E. pauciflora* from subgenus *Eucalyptus*) that differ qualitatively in their chemical profiles. Trees of each subgenera contain a wide range of terpenes and phenolic compounds, but only species from the subgenus *Symphyomyrtus* contain structurally complex FPCs such as sideroxylonals and macrocarpals (Eschler et al., 2000).

In this work, we tested the hypothesis that elevated [CO_2] would alter concentrations of essential oils and phenolic compounds in *Eucalyptus* foliage as demonstrated in other species (Lindroth et al., 1993; Agrell et al., 2000; Knepp et al., 2005; McElrone et al., 2005). Consequently, the aims of this research were to determine: 1) if elevated [CO_2] affected the concentration of foliar primary and secondary chemical traits in *E. globulus* and *E. pauciflora* seedlings; 2) if there was strong genetic control of variation in leaf chemistry of either species; and 3) if there was a genotype by environment ([CO_2]) interaction whereby elevated [CO_2] affected PSM concentrations differently among populations within a species.

Methods and Materials

Population Selection and Germination Using the same facilities for each eucalypt species, data in this paper were collected in two complementary experiments that tested *E. globulus* in 2007, and *E. pauciflora* in 2010. Growth environment, sample preparation, and analysis varied between species; however, traits were not compared between species. Open pollinated seed was collected from individual trees (5–10) from native *E. globulus* (3) and *E. pauciflora* (10) populations. *Eucalyptus globulus* populations were selected based on variation in defensive chemical properties (O'Reilly-Wapstra et al., 2004, 2007). Pooled seed from a single tree was designated a 'family'. A total of 15 families were selected from three *E. globulus* populations (Table 1). As no previous study had assessed variation in chemistry among *E. pauciflora* populations, 10 geographically distant

Table 1 Number of families, altitude, latitude and longitude of each population for the two eucalypt species used in this study

Species	Population	No. of families	Altitude (m)	Latitude	Longitude
E. globulus	Strzelecki	5	600	38°24′	146°28′
	Blue Gum Hill	5	312	43°03′	146°53′
	St Helens	5	118	41°16′	148°18′
E. pauciflora	Great Lake	10	1058	42°01′	147°49′
	Lake Arthur	9	1002	41°58′	146°51′
	Lake St Clair	9	817	42°11′	146°10′
	Rossarden	7	732	41°41′	147°41′
	Lake Leake	9	609	42°01′	147°49′
	Lake Rowallan	8	467	41°43′	146°13′
	Oatlands	8	327	42°18′	147°22′
	Longford	10	159	41°37′	147°05′
	South Arm	7	19	43°01′	147°25′
	Waterhouse	5	28	40°53′	147°39′

E. pauciflora populations were selected. A total of 82 *E. pauciflora* families were selected from the 10 populations (Table 1).

Seeds from each *E. globulus* family (15 families) were wet stratified at 3°C for 3 wk, then germinated in a controlled environment glasshouse. Germinants were grown for 1 mo, then uniform size individuals (>8 germinants per family depending on germination rate) were transplanted into individual plastic tubes (base 38×38 mm, top 50×50 mm, height 118 mm). Half of the germinants from each family were allocated to the elevated [CO_2] treatment, and half to the control. Germinants in each group were arranged randomly into replicates. The control group germinants were placed in an individual controlled environment glasshouse, while the elevated [CO_2] treatment germinants were placed in a second adjacent and identical glasshouse.

Seeds from each *E. pauciflora* family (82 families) were wet stratified at 3°C for 2 wk, then germinated. Germinants were grown for 1 mo, and then uniform size individuals were transplanted into individual tubes as described above (5–18 germinants per family depending on germination rate). Half of the germinants from each family were allocated to the elevated [CO_2] treatment, and half to the control (586 seedlings per treatment). All germinants within each treatment were completely randomized (i.e., not in families) then arranged into replicates (15 replicates per treatment). The control group was placed in a controlled environment glasshouse. An elevated [CO_2] treatment was applied to the second group in an adjacent identical glasshouse.

Potting mix and nutrient applications varied between eucalypt species, due to differences in growth requirements and because peat moss (used for *E. pauciflora* in the second trial) was found to be more suitable as a potting medium than pine bark (used for *E. globulus* in the first trial). Potting mix used for *E. globulus* contained eight parts composted fine pine bark: three parts course river sand, and N:P:K [19: 2.6: 10] at 1 g /L potting mix. The pH was adjusted to approximately 6 with the addition of dolomite lime at 3 kg/m^3. Potting mix used for *E. pauciflora* contained one part peat moss to three parts sand, with fertilizer (8–9 month Osmocote® N:P:K 17:1.6:8.7) at 1 g /L potting mix. The pH was adjusted to approximately 6 with the addition of dolomite lime at 2.7 kg/ m^3. Low concentration fertilizer (Peters Excel 13N:2.2P:16.6K) was applied infrequently to seedlings of both species at 0.5 g per litre of water to maintain growth. Chelated iron was added twice to *E. pauciflora* at 2.5 ml per 9 L due to iron deficiency symptoms. Iron was not applied to *E. globulus* seedlings as iron deficiency was not apparent. Seedlings in both experiments were watered daily, and harvested after 5 mo.

Elevated Carbon Dioxide Treatment The average background [CO_2] in the urban glasshouse complex was 450 ppm, which is above the current atmospheric [CO_2]. Therefore, the elevated [CO_2] treatment applied to both species was 630 ppm, with actual levels fluctuating between 620–650 ppm. The [CO_2] was automatically maintained by using a CO_2 control unit (Thermoline Scientific Equipment, Australia) and compressed CO_2. The CO_2 was delivered to seedlings via the glasshouse air recirculation system, and no stream of CO_2 directly hit the plants. Glasshouse [CO_2] concentration was measured twice weekly at multiple points within the glasshouse at plant height. Seedlings and designated treatments of both the *E. globulus* and *E. pauciflora* experiments were rotated weekly between and within glasshouses to remove non-random environmental effects. Extensive measurement of leaf gas exchange of both species has indicated that the light saturated rate of carbon assimilation in these species does not saturate until an external [CO_2] of above ~750 μmol CO_2 mol^{-1} air, making the concentrations used below the inflection point on the photosynthetic CO_2-response curve for both these species. Thus, the CO_2 treatments used would have had pronounced impacts upon leaf gas exchange.

Sample Collection Within each treatment of the *E. globulus* seedlings, fully expanded leaves (3–4 leaf pairs per seedling) from all seedlings from a single family were pooled into a single sample (15 family samples in each treatment). Leaves were pooled to produce adequate amounts of leaf material for multiple analyses. Each family sample was mixed and divided into duplicate samples (except one due to lack of foliage). From each of the 59 samples, 2–3 g of leaves were randomly sub-sampled for essential oil analysis. The remaining leaves were freeze dried and ground for analysis of primary chemistry, FPCs, and other phenolic compounds.

Eucalyptus pauciflora seedling heights (soil surface to base of apical bud) were measured in week 14 of the experiment (82 families). Families containing fewer than three seedlings were excluded from chemical analyses. Fully expanded leaves (2–3 leaf pairs per seedling) of all seedlings from a single family (3–9 seedlings per family) were picked and pooled to give one bag of foliage per family (75 families). From the pooled family leaf samples, 1–2 g of leaf were randomly sub-sampled, freeze dried, and ground for analysis of primary chemistry. Remaining pooled leaves which totaled 2–3 g (98 samples) were used for oil analysis.

Primary Chemistry Freeze dried leaf samples (150 *E. pauciflora*, 59 *E. globulus*) were ground to a fine powder using a ball mill (Retsch MM200) and analyzed for carbon, nitrogen, and hydrogen content. Due to the time between experiments, *E. globulus* samples were analyzed for C, H, and N content using a Perkin Elmer 2400 Series II elemental analyzer, while *E. pauciflora* samples were analyzed using a newly acquired Thermo Finnigan EA 1112 Series Flash Elemental Analyzer.

Secondary Chemistry For *E. globulus,* we assayed two FPCs (sideroxylonal-A and macrocarpal-G, the two dominant isomers of these compounds in *E. globulus*), total phenolics, total oil yield, and 1,8-cineole (the dominant monoterpene in *E. globulus*). For *E. pauciflora*, we focused on comprehensively investigating the essential oil (total oil yield and 18 specific oil components). The FPCs were assayed by high performance liquid chromatography (HPLC) following methods of Wallis and Foley (2005). Sideroxylonal-A was expressed as mg g DM^{-1}, while macrocarpal-G was expressed as mg g DM^{-1} equivalents of macrocarpal-A, using standards described by Eyles et al. (2003). Total phenolics were assayed with the modified Prussian blue assay (Graham, 1992). Foliage was prepared and extracted following the method outlined in Hagerman (2002). Using a commercial gallic acid standard (Sigma G-7384), the concentration of total phenolics was expressed as mg g DM^{-1} equivalents of gallic acid.

Oils were extracted using a method modified from O'Reilly-Wapstra et al. (2004). Frozen leaf samples were cut into 5 mm squares, disregarding the midrib and damaged regions, and 1–2 g of leaf were extracted in a dichloromethane (DCM) stock solution containing 100 μg L^{-1} of n-heptadecane as an internal standard. The DCM solution (10 ml) was added to tubes containing leaf squares, then refrigerated overnight (*E. pauciflora*) or for 60 min (*E. globulus*). Tubes were sonicated for 30 min and vortex mixed, before transferring the extract to a new tube. Samples were extracted two more times with 5 ml (*E. pauciflora*) or 10 ml (*E. globulus*) of DCM, refrigerated for 60 min, sonicated for 30 min, and vortex mixed before removing the extract and pooling it with the first extract. The *E. pauciflora* extracts (20 ml) were evaporated to 4 ml because the oil content of these samples was low relative to internal standard (1.96 mg g DM^{-1} cineole equivalents). *Eucalyptus globulus* extracts did not require concentration because the mean oil content was significantly higher (36.41 mg g DM^{-1} cineole equivalents). The extraction procedure was carried out for 98 *E. pauciflora* samples (fewer than primary chemistry due to limited leaf material), and 59 *E. globulus* samples.

Eucalyptus globulus extracts were characterized and quantified using a Varian 3800 gas chromatograph coupled to a Varian 1200 triple quadrupole mass spectrometer as described in O'Reilly-Wapstra et al. (2011), while essential oil components in *E. pauciflora* samples were first identified with GC/MS as above, then quantified with a newly acquired Varian 450-GC/FID using the same column and conditions as above but with nitrogen as carrier gas, using Varian Galaxie software. Individual compounds were identified through a combination of the NIST Mass Spectral database (NIST/EPA/NIH mass spectral library, NIST 08, August 2008, US Department of Commerce), a comprehensive in-house database of essential oil components including beta-triketones, and Kovats' retention indices (Davies, 1990) (Supplemental Table 1). Results were expressed as mg g DM^{-1} for 1,8-cineole and α-pinene using standards, while total oil yield and other oil components were expressed as mg g DM^{-1} cineole equivalents. Compound concentrations from both species were quantified in 2007 and 2010 using the same standards. Some essential oil components were very low in certain *E. pauciflora* populations and at the limit of detection with the methods used (e.g., 0.001 mg g DM^{-1} cineole equivalents).

Statistical Analysis The two experiments were analyzed separately. One *E. pauciflora* population (Waterhouse) was excluded from oil analysis as no samples were harvested from the elevated [CO_2] treatment. All analyses were completed using SAS statistical software package (version 9.2, SAS Institute Inc., Cary, NC, USA). Treatment (elevated [CO_2], control), population (3 *E. globulus* populations, 9 or 10 *E. pauciflora* populations) and interactions were analyzed for each variable using general linear model procedures (PROC GLM). *Eucalyptus globulus* chemical traits (5), and *E. pauciflora* essential oil components (13) were analyzed for treatment, population, and interaction effects using multivariate analysis (PROC MANOVA), followed by discriminant analysis (PROC DISCRIM). Three *E. pauciflora* populations (South Arm, Rossarden and Lake Leake) were omitted from multivariate and discriminant analysis due to low replication. Variables were checked for assumptions of normality and heterogeneity and transformed where necessary. *Eucalyptus pauciflora* total oil, α-pinene, β-pinene, limonene, *trans*-β-ocimene, α-terpineol, caryophyllene, and robustaol-B (tentatively identified) values were transformed to their natural logarithm. *Eucalyptus pauciflora* myrcene, aromadendrene, 1,8-cineole, bicyclogermacrene, unknown phenolic compound-A, and unknown phenolic compound-B were transformed to their square roots. *Eucalyptus pauciflora* torquatone, cymene, elemol, β-eudesmol, and α–phellandrene data consisted of many zero values due to their abundances falling below the limit at which we could accurately quantify (more than 40% zero values), and hence, did not fit a normal distribution. These five traits were analyzed individually by comparing all possible pairings of *E. pauciflora* populations using Kruskal-Wallis non-parametric tests (PROC NPAR1-WAY), and excluded from multivariate analysis. Transformation was not required for *E. pauciflora* height data or for *E. globulus* variables.

Results

Primary Chemistry Elevated [CO_2] had little effect on leaf primary chemistry in either eucalypt species (Table 2), increasing only *E. globulus* leaf carbon content significantly

Table 2 Results of the general linear model (GLM) analysis for variation in primary chemistry of *Eucalyptus pauciflora* and *Eucalyptus globulus* seedlings at the population, treatment, and population x treatment interaction levels

Species	Primary chemistry	Population		CO_2 treatment		Population x CO_2 treatment interaction	
		$F_{2,58}$	P	$F_{1,\ 58}$	P	$F_{2,58}$	P
E. globulus	Carbon	1.11	0.34	5.6	0.02	0.72	0.49
	Hydrogen	0.56	0.57	0.77	0.39	1.23	0.3
	Nitrogen	3.96	0.03	0.47	0.5	0.21	0.81
		$F_{9,149}$	P	$F_{1,149}$	P	$F_{9,149}$	P
E. pauciflora	Carbon	4.86	<0.0001	0.34	0.56	1.33	0.23
	Hydrogen	2.14	0.03	0.01	0.92	1.17	0.32
	Nitrogen	1.46	0.17	0.09	0.77	0.67	0.73

(P=0.02) (Supplemental Table 2). Population level variation in some primary chemical traits was detected within both eucalypt species; however, patterns were not consistent between species (Table 2). More specifically, carbon (P<0.001) and hydrogen (P=0.03) varied significantly between *E. pauciflora* populations; while nitrogen (P=0.03) content differed between *E. globulus* populations (Table 2). There were no population by treatment interactions for either species, indicating that population level differences in primary chemistry were stable across the two [CO_2] treatments.

Secondary Chemistry There were no significant differences in levels of individual essential oil components (including terpenes), total oil yield, total phenolics, condensed tannins, sideroxylonal-A, or macrocarpal-G between the two [CO_2] treatments (Table 3 and Supplemental Table 2). In contrast, highly significant differences in the concentrations of many individual PSMs were detected between *E. globulus* populations, and between *E. pauciflora* populations (Table 3). *Eucalyptus globulus* mean total oil yield was 36.41 mg g DM^{-1} cineole equivalents. For *E. globulus*, foliage from the St Helens population contained the lowest quantities of many metabolites, while Blue Gum Hill contained the highest concentrations of macrocarpal-G, and Strzelecki contained the highest concentrations of sideroxylonal-A (Supplemental Table 3). *Eucalyptus pauciflora* mean total oil yield was 1.96 mg g DM^{-1} cineole equivalents. The dominant terpene within *E. pauciflora* oil was α-pinene, although the Rossarden population contained 1,8-cineole as the dominant terpene (Supplemental Table 4). The Longford population contained the lowest total oil yield of all *E. pauciflora* populations (0.51 mg g DM^{-1} cineole equivalents), along with the lowest concentrations of many oil components. The South Arm population contained the most total oil (4.36 mg g DM^{-1} cineole equivalents). Three oil components could not be positively identified, and are referred to here as robustaol-B (tentatively identified), unknown phenolic compound-A (a β-triketone or phenol, with a prominent ion at m/z 168), and unknown phenolic compound-B (a β-triketone or acyl-phenol, with prominent ions at m/z 224, and 167). No population by treatment interactions were detected for chemical traits in either eucalypt species (Table 3), indicating that population level differences in secondary chemistry were stable across the two [CO_2] treatments.

Similar to the univariate analyses, variation (P<0.001) in the five *E. globulus* chemical traits was detected among populations using multivariate analysis. No treatment effect or interaction was discovered. Discriminant analysis and ordination using five *E. globulus* chemical traits show clear grouping of the populations with minimal treatment effect (Fig. 1). Multivariate analysis of *E. pauciflora* oil components revealed population (P<0.001) and treatment effects (P=0.04). There was no *E. pauciflora* population x treatment interaction detected indicating that there was no difference in the population responses to the [CO_2] treatment. Discriminant analysis and ordination of the *E. pauciflora* essential oil components illustrate population and treatment effects (Fig. 1).

Growth Eucalyptus pauciflora seedling heights were different among populations (P<0.001). There was also a treatment effect (P=0.009), with control seedlings (least squares mean 57.18 mm) taller than the elevated [CO_2] treatment seedlings (least squares mean 52.26 mm) at 14 weeks. No significant interaction between population and treatment was detected.

Percentage Dry Matter (%DM) The %DM of *E. globulus* foliage was not significantly different among populations or treatments, and no population by treatment interaction was identified. A significant difference was identified in %DM of *E. pauciflora* leaves among populations (P=0.03). There was no significant difference in *E. pauciflora* foliage %DM between treatments, and no significant population by treatment interaction was identified.

Discussion

The past decade has seen an increase in literature that examines elevated [CO_2] effects on PSMs across many plant

Table 3 Results of the general linear model (GLM) analysis for variation in *Eucalyptus pauciflora* seedling oil components along with *Eucalyptus globulus* chemical traits at the population, treatment (elevated [CO_2] and control), and population X treatment interaction levels

Species	Chemical trait	Population effect		CO_2 treatment effect		Population x CO_2 treatment interaction	
		$F_{2,58}$	P	$F_{1,\ 58}$	P	$F_{2,58}$	P
E. globulus	Sideroxylonal-A[a]	22.77	<0.001	0.91	0.34	0.07	0.93
	Macrocarpal-G[a]	19.93	<0.001	0.42	0.52	0.64	0.53
	Total Phenolics	0.54	0.59	0.05	0.82	0.35	0.71
	1,8-cineole	20.73	<0.001	0.29	0.59	1.48	0.24
	Total oil	13.61	<0.001	0.31	0.58	1.71	0.19
		$F_{8,97}$	P	$F_{1,97}$	P	$F_{8,97}$	P
E. pauciflora	*α –pinene*[b]	11.25	**<0.001**	0.08	0.77	1.28	0.26
	β -pinene[b]	3.91	**<0.001**	0.08	0.78	0.81	0.59
	Myrcene[c]	11.3	**<0.001**	0.04	0.84	1.14	0.35
	limonene[b]	11.31	**<0.001**	0.00	0.95	0.51	0.84
	1,8-cineole[c]	15.35	**<0.001**	0.11	0.74	1.47	0.18
	trans-β-ocimene[b]	3.77	**0.001**	0.54	0.47	0.54	0.82
	α-terpineol[b]	7.33	**<0.001**	0.49	0.49	0.65	0.74
	caryophyllene[b]	1.53	0.16	0.38	0.54	1.11	0.39
	aromadendrene[b]	1.74	0.11	0.06	0.81	0.55	0.79
	bicyclogermacrene[b]	5.26	**<0.001**	1.95	0.17	1.19	0.32
	robustaol-B[b,d]	6.00	**<0.001**	0.3	0.59	1.47	0.18
	Unknown-A[c,e]	8.94	**<0.001**	1.00	0.32	0.48	0.87
	Unknown-B[c,e]	9.17	**<0.001**	0.23	0.63	0.75	0.64
	Total oil[b]	12.07	**<0.001**	0.25	0.62	1.17	0.33
		x^2_8	P	x^2_1	P		
	α -phellandrene[f]	40.0	**<0.001**	2.28	0.13		
	cymene[f]	47.82	**<0.001**	1.31	0.25		
	elemol[f]	31.45	**0.001**	0.12	0.73		
	β-eudesmol[f]	9.61	0.29	1.27	0.26		
	Torquatone[d,f]	44.25	**<0.001**	0.05	0.82		

Bold type indicates significance of *E. pauciflora* traits after Bonferroni adjustment ($P<0.003$). Italics indicate monoterpene, standard font indicates sesquiterpene.

[a]indicates FPC

[b]indicates data which required log transformation

[c]denotes data which was squared

[d]indicates β-triketones (non-terpene component)

[e]indicates unknown phenol (non-terpene component)

[f]denotes data analysed by Kruskal-Wallis non-parametric tests where interaction terms were not analysed due to zero inflated data and low chemical concentrations

taxa (Kuokkanen et al., 2004; Mattson et al., 2004; Bidart-Bouzat and Imeh-Nathaniel, 2008). However, few studies have examined the effects of CO_2 on the species rich, ecologically and commercially important *Eucalyptus* genus (Lawler et al., 1997; Gleadow et al., 1998). Here, we show that elevated [CO_2] generally did not affect concentrations of PSMs in seedlings of two eucalypt species from different subgenera. Furthermore, elevated [CO_2] did not affect the stability of genetically based differences in concentrations of compounds in these species.

Patterns of phenolics are often particularly plastic in response to changing [CO_2] (Lindroth, 2010). However, we did not detect changes in the phenolic-based compounds (total phenolics, tannins, and FPCs) of *E. globulus* seedlings. Lawler et al. (1997) showed that phenolic concentrations in leaves of *E. tereticornis* seedlings increased under elevated [CO_2], but only in conjunction with certain nutrient and light regimes. We did not manipulate soil nutrient and light conditions. The lack of effect of elevated [CO_2] on phenolics in our study and the specific interactive effects needed to elicit a response in seedlings demonstrated by Lawler et al. (1997) suggest that elevated [CO_2] alone does not have a strong, consistent effect on the concentration of leaf phenolics in eucalypt seedlings.

Resource limitation can restrict or eliminate the response of plants to elevated [CO_2] (McMurtrie et al., 2008; Lindroth, 2010). For example, the magnitude of [CO_2] effect on *Pinus taeda* net primary productivity is determined by nitrogen and water availability (McCarthy et al., 2010). Combined nutrient and elevated [CO_2] manipulations have been shown to elicit a response in some PSMs in eucalypts (for example total phenolics), when elevated [CO_2] alone did not (Lawler et al., 1997). Our *a priori* hypothesis was that elevated [CO_2] would alter the concentration of terpenes, phenolic compounds, and the carbon content of leaves. We used low to moderate nutrient medium to simulate natural Australian soil conditions. Perhaps the seedlings grown under elevated [CO_2] in this study were nutrient

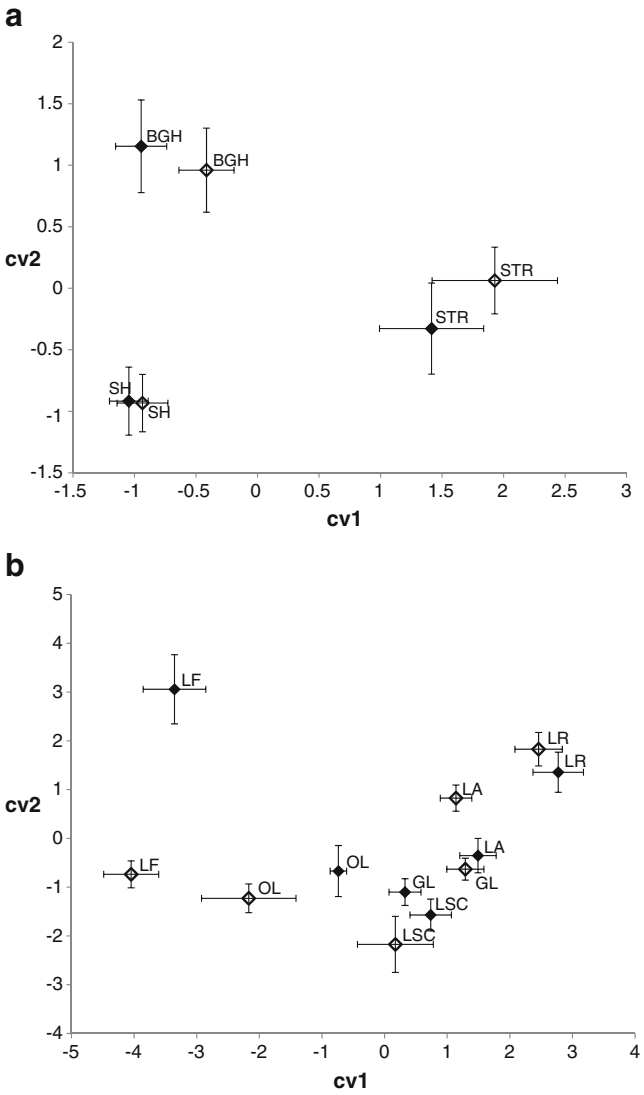

Fig. 1 Response of leaf chemical traits in seedlings from (**a**) *Eucalyptus globulus* and (**b**) *E. pauciflora* populations grown under elevated carbon dioxide or control treatments. Open symbols indicate elevated [CO_2] treatment, closed symbols indicate control. *Eucalyptus globulus* ordination based on sideroxylonal-A, macrocarpal-G, total phenolics, 1,8-cineole and total oil concentrations. *Eucalyptus pauciflora* ordination based on α-pinene, β-pinene, limonene, *trans*-β-ocimene, α-terpineol, caryophyllene, robustaol-B (tentatively identified), myrcene, aromadendrene, 1,8-cineole, bicyclogermacrene, unknown phenolic compound-A, and unknown phenolic compound-B concentrations. *BGH* Blue Gum Hill, *SH* St Helens, *STR* Strzelecki, *LF* Longford, *OL* Oatlands, *LSC* Lake St Clair, *GL* Great Lake, *LA* Lake Arthur, *LR* Lake Rowallan

limited, and therefore, unable to respond to the increase in available carbon. Given the inherently low nutrient status of Australian soils (Attiwell and Leeper, 1987), the effects of elevated [CO_2] on wild germinating seedlings of *Eucalyptus spp.* may be reduced compared with eucalypt seedlings grown in nutrient rich regions of the world (Hovenden and Williams, 2010). In addition, the eucalypt species in this study exhibit clear heteroblastic life stages, and we are unsure if the consistent lack of response in seedling foliage to elevated [CO_2] detected in this study would continue across further life stages. Our previous work indicated that genetically based differences in the expression of FPCs from adult to juvenile coppice foliage were stable in *E. globulus* (O'Reilly-Wapstra et al., 2007) suggesting that the response of PSMs to increased [CO_2] also may be stable, but further investigation is required.

The majority of studies that examine the effects of [CO_2] on PSMs focus on major, broad compound groups without examining the effects on individual compounds. Such an approach may mask responses of individual compounds. We focused on 18 individual compounds in *E. pauciflora* essential oil. In the univariate analysis, we did not detect any response of individual terpenes, other oil components, or total oil yield to elevated [CO_2]. Similarly, elevated [CO_2] did not change total oil content in *E. globulus*. Our results are consistent with those obtained previously for *E. tereticornis* (Lawler et al., 1997). Elevated [CO_2] also has been shown to have little effect on terpene concentrations in some Northern Hemisphere species (Lindroth, 2010), indicating that stability of terpene concentrations regardless of [CO_2] may be widespread.

Elevated [CO_2] decreased *E. pauciflora* seedling height irrespective of genotype. Hence, responses to elevated [CO_2] may have been expressed in our seedlings through other traits, rather than through effects on PSMs. Alternative responses in eucalypts could include increases in both root and shoot biomass (Atwell et al., 2009). Evidence for carbon allocation to below ground sinks under elevated [CO_2] has been observed in diverse tree species (Griffin et al., 1997; Thomas et al., 1999). We did not investigate above- or below-ground biomass in these eucalypts. However, as seedling height decreased in the elevated [CO_2] treatment, it is unlikely that shoot biomass increased. The next stage in understanding the effects of elevated [CO_2] on eucalypts is to investigate the responses of both foliar secondary chemical concentrations and below-ground carbon allocation to elevated [CO_2], preferably while under different levels of resource availability such as nutrients, water or light.

The concentrations of PSMs in eucalypt seedlings may be stable in relation to future elevated [CO_2]. Desirable chemical traits selected by resource managers and the extended effects of these compounds throughout the associated community and ecosystem may also be stable under future [CO_2] conditions, at least during the early stages of eucalypt life history. However, elevated [CO_2] may alter PSM concentrations of eucalypt seedlings when acting in conjunction with other environmental factors.

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Volatile Profile Differences and the Associated *Sirex noctilio* Activity in Two Host Tree Species in the Northeastern United States

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Abstract *Sirex noctilio* females are known to be attracted to stem sections of stressed pine trees for oviposition. The volatile profiles and attractiveness of Eastern white pine (*Pinus strobus*) and two chemotypes of Scots pine (*P. sylvestris*) were compared after stem injection with herbicide. In general, trap captures on herbicide-treated trees were higher than on controls. The high-carene chemotype of Scots pine captured the highest numbers of females, followed by the low-carene chemotype, and finally the Eastern white pine. Herbicide-treated trees of both species emitted larger quantities of volatiles than the controls. The herbicide treatment induced higher volatile emission rates in the Scots pine chemotypes than in white pine, although there was no difference between the two chemotypes. However, qualitative differences were found between the volatile profiles of the two species as well as between the two Scots pine chemotypes, which could account for the differential attractiveness of the species and chemotypes tested.

Keywords *Sirex noctilio* · Lure development · Stem injection · High-carene Scots pine · Host volatiles · Hymenoptera · Invasive insect

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Introduction

Plants produce a wide array of volatile compounds by which they lure pollinators and natural enemies of their herbivorous pests (Bruce and Pickett 2011). However, these compounds frequently serve as host finding cues for the herbivores themselves. Having co-evolved with their plant hosts, insects have developed an olfactory system that houses olfactory receptor neurons (ORNs) finely tuned for specific volatile compounds (Hansson et al. 1999). At any time, insects encounter blends of volatiles and receive numerous components simultaneously at the peripheral level, but the behavioral output, i.e., directed flight towards or away from a source and avoidance or acceptance, is formed in higher centers of the brain (Baker 2009).

Wood-feeding insects also rely on volatile compounds emitted by host and non-host tree species to make the right choice for landing, although it varies to which extent primary attraction plays a role in pre-landing behavior (Saint-Germain et al. 2007). While conifer monoterpenes are important cues in host finding behavior of bark beetles (Gijzen et al. 1993), in some species integration of visual and olfactory cues is necessary for successful assessment of the potential host (Campbell and Borden 2009).

In our study system, the woodwasp *Sirex noctilio* F. (Hymenoptera: Siricidae), females disperse soon after emergence (Morgan and Stewart 1966) and find their preferred host for oviposition, mostly *Pinus* species (Hurley et al. 2007). As many other wood-feeding insects, *S. noctilio* females are attracted to physiologically stressed trees: the stem of suppressed pines, stem sections conditioned by caged females (Madden 1968), or artificially stressed pine trees (Zylstra et al. 2010). Females possess a ventral ovipositor in a sheath modified for inserting eggs about a centimeter deep into the wood of their host (Schiff et al. 2006). With each drill, whether just probing the wood or ovipositing, females inject mucus and the arthrospores of the symbiotic fungus, *Amylostereum aerolatum* (Coutts and Dolezal 1969). The fungus is essential for the development of the larvae. In areas where *S. noctilio* populations are dense, successive attacks may result in the death of healthy trees (Morgan and Stewart 1966) due to the combined effects of the mucus and the fungus (Spradbery 1973).

Host finding behavior of a female *S. noctilio* could be viewed as a three-step process: 1) terminating the search flight and landing on a possible host, i.e., prealighting behavior (Singer 1986); 2) drilling into the wood to assess wood quality; and 3) laying eggs if the tree is accepted as a suitable host. We have been investigating the volatile compounds that may determine the behavior of the woodwasp in the first step.

According to Madden (1968), the physiological changes that occur in the host upon the introduction of natural and artificial stressors are similar. Interrupted translocation of soluble solids and increased respiratory activity of the phloem tissues lead to depletion of food reserves. After high mechanical girdling, for example, the stem section below the treatment becomes attractive to females. The diffusion rate of carbon dioxide as well as of water vapor and monoterpenes through that stem section becomes more rapid compared to healthy parts (Madden 1968, 1988). Attraction of female woodwasps to stressed trees has been attributed to enhanced emissions of monoterpenes (Madden 1988).

Although monoterpene constituents of the resin of Monterey pine (*Pinus radiata*), such as α-pinene, β-pinene, δ-3-carene, β-myrcene, and β-phellandrene, elicit strong electrophysiological response in the antenna of females (Simpson 1976), chemical lures containing these compounds individually or in combination have not been as successful as herbicide-injected lure trees in capturing the insect in the Northeastern United States (unpublished data of the authors).

Sirex noctilio is an invasive pest in North America (Hoebeke et al. 2005; Hurley et al. 2007). Preferred host species in the current *S. noctilio* range on this continent are the native Jack pine (*P. banksiana*), red pine (*P. resinosa*), and Eastern white pine (*P. strobus*), and the introduced Scots pine (*P. sylvestris*) (Dodds et al. 2010). In our trapping study conducted in the summer of 2008 over ten sites, Scots pine and white pine captured much higher numbers of *S. noctilio* females when conditioned by stem-injection with the herbicide Dicamba (3,6-dichlororo-2-methoxybenzoic acid) compared to untreated controls (unpublished data of the authors). In addition, there was a strong trend with herbicide-treated (HT) Scots pine capturing higher numbers of woodwasps than HT white pine. Volatile compounds were collected from trap trees at only one of the ten sites. Herbicide treatment caused increased volatile emissions in both species, however, more so in Scots pine (unpublished data of the authors). These results prompted us to perform a field trapping experiment combined with collection of volatiles emitted by trap trees of the two pine species.

Our goal in this study, conducted in 2009, was to investigate volatile production and simultaneously monitor trapping efficiency of two host species of *S. noctilio*, Scots pine (*P. sylvestris*) and white pine (*P. strobus*), during the flight season. The high-carene and low-carene producer chemotypes of Scots pine (Chalchat et al. 1985) were included as two different treatment groups. We developed a system to collect volatiles from stem sections of living trees causing minimal disturbance, which allows us to monitor the volatile production of the same trees throughout the trapping season. Based on our findings we suggest the testing of specific compounds with electrophysiological methods and in behavioral assays to identify chemicals that mediate host finding behavior in *S. noctilio* and possibly facilitate the development of a chemical lure.

Methods and Materials

Experimental Setup and Trapping Mixed sites with both Scots pine and white pine were selected in the Syracuse area of New York. Only trees with no signs of infestation were used in the experiments. An attempt was made to keep uniformity of height, stem diameter, and foliage. Trees of the same treatment were grouped in clumps of three within about a 5 m circle to enhance trapping efficiency. For a simple overview of the experimental setup see Table 1.

Our treatment groups reflected the fact that Scots pine occurs in two chemotypes. Scots pine chemotypes were determined by analyzing the resin composition before the start of the season. Resin was collected from Scots pine trees (153 trees altogether) at four potential sites in May by inserting a 1-ml screw cap vial into a 2–3 cm deep hole drilled in the stem at 45°, and the samples were analyzed for δ-3-carene (see Sample Preparation and Chemical Analysis). Three sites were found to have sufficient numbers of both chemotypes and were selected for simultaneous

Table 1 Experimental setup

Treatment groups	High-carene Scots pine: HT and control
	Low-carene Scots pine: HT and control
	White pine: HT and control
Nr. of sites	3
Nr. of replicates within site	3 trees per clump[a]
	1 clump of each HT group per site
	1 clump of each control group per site
Frequency of trap checking	Weekly
Type of traps used	Multiple-funnel traps
Arrangement of traps	1 trap per tree
Volatile collection time-points	Week 0, 3, 4, 5, 6, 7, 8, 9, 11

HT herbicide-treated, control: untreated

[a] Except for low-carene Scots pine: one of the sites had only two trees in the HT and two in the control clump due to herbicide treatment of the wrong tree and misidentification of the chemotype, respectively

trapping and volatile collection. The treatment groups were: herbicide-treated (HT) and control high-carene Scots pine, HT and control low-carene Scots pine, and HT and control white pine.

In summary, we had three sites each of which had one clump for each of the six treatment groups, and each clump had three trees (except at one site the low-carene HT and control clump had only two trees, due to herbicide-injection of the wrong tree in the HT clump and misidentification of the chemotype in the control clump). Each tree in the study had a multiple-funnel trap attached (Lindgren 1983). Thus, the number of replicates was 9 for HT and control high-carene Scots pine and white pine, and 8 for HT and control low-carene Scots pine. Stem injection of Dicamba to stress the HT trees was performed in the week of June 8 (week 0) according to the methods described by Zylstra et al. (2010). Traps were checked weekly between the weeks of June 29 (week 3) and Sept 21 (week 15). Volatiles were collected from all HT and control trees first in week 0, then every week between week 3 and week 9, and finally in week 11. Volatiles were not collected on rainy days.

At the end of the trapping season, one tree from each treatment group was felled and bucked into equal-sized billets and brought back to the USDA laboratory in Syracuse, NY. The ends of all of the billets were coated with Waxlor (Willamette Valley Co., Eugene, OR, USA) to prevent drying. Billets from the same tree were placed into drums with screens and maintained at 26°C and <20% RH. Overhead fluorescent lights were turned on at 7:30 am and turned off at 4:30 pm. Once emergence commenced, siricids were collected, identified, and tallied daily until no more *Sirex* individuals were emerging from the material.

Volatile Collection A portable two-pump volatile collection system with a teflon-sheet chamber (FEP100 fluoropolymer film; Dupont, Wilmington, DE, USA) was developed to sample sections of a living tree without inflicting damage (Fig. S1 in Supplemental Information). The collection chamber is constructed from a wire frame wrapped with a FEP sheet and secured to the trunk with straps. Two grommet-reinforced holes in the FEP film located on opposite sides of the tree allowed us to introduce purified air on one side and install a filter filled with 30 mg of SuperQ sorbent (Alltech Associates, Deerfield, IL, USA) (SuperQ filter) on the opposite side to collect volatiles. One pump pushed air into the chamber through a charcoal filter, and the other pulled air out of the chamber through the SuperQ filter.

Volatiles were collected for 30 min at a flow rate of 0.6–0.7 l/min measured through the SuperQ filter. The ingoing flow rate of purified air was set to 1.0–1.2 l/min. Flow rates were checked with variable area flow meters (Key Instruments, Trevose, PA, USA) and adjusted if needed every morning. Before each collection 1 μl of a solution containing 100 mg/ml of 1,4-diisopropyl benzene in hexane was added to a small glass cartridge filled with glass-wool. The glass cartridge was inserted at the end of the ingoing air tube. This compound was an indicator of pump performance and air flow. Filters on which volatile compounds had been collected were sealed with Teflon® tape at both ends, wrapped in aluminum foil, and kept at room temperature until analysis (typically for 1–2 weeks). Filters were washed with a sequence of acetone, dichloromethane, and hexane, and were stored in aluminum foil packages between experiments.

Sample Preparation and Chemical Analyses All samples were taken to the Penn State laboratory for analysis. For the determination of the Scots pine chemotype, a small volume (5–10 μl) of resin was diluted in 500 μl of hexane (Mallinckrodt Baker, Phillipsburg, NJ, USA), and the sample was analyzed under the same conditions as the volatile collection samples (see below). Resin samples were only examined for the presence of δ-3-carene based on comparison of the retention time to that of the purchased reference. SuperQ filters were eluted with 150 μl of a solution containing 50 μg/ml of nonyl actetate (internal standard) dissolved in a 1:1 mixture of hexane and dichloromethane (Burdick & Jackson, Morristown, NJ, USA). Nitrogen flow was applied onto the filters to assist elution.

All samples were analyzed in an Agilent 6890 gas chromatograph-flame ionization detector system (GC-FID) equipped with an Equity-5 (30 m×0.2 mm×0.2 μm; Supelco, Bellefonte, PA, USA) column. Helium was used as carrier gas at an average linear velocity of 25 cm/s. Samples were injected in the splitless mode, and the injector was changed to split mode after 0.75 min. The initial oven temperature was held at 45°C for 1 min, then programmed

to 300°C at a rate of 10°C/min, and maintained at that temperature for 10 min. The injector and the detector temperatures were set to 280°C and 300°C, respectively. The enantiomeric ratio of α-pinene was determined by injecting samples on an Rt-βDEX (30 m×0.25 mm×0.25 μm; Restek, Bellefonte, PA, USA) column installed in the above mentioned GC-FID. The oven program was the following: 45°C (1 min hold)-3°C/min to 140°C, then 10°C/min to 210°C (10 min hold). The injector and the detector were both kept at a temperature of 210°C. Other parameters were the same as above.

To identify blend components selected samples were analyzed in a GC-MS system consisting of an Agilent 6890 N gas chromatograph interfaced with an Agilent 5973 N mass selective detector. The capillary column was equivalent to that used in the GC-FID. Helium was used as carrier gas at an average linear velocity of 30 cm/s. Samples were injected in splitless mode with a split time of 0.75 min. Temperature settings were the same as in the GC-FID, except that here the transfer line was held at 300°C. The MS was used in electron impact (EI) ionization mode with the default temperature settings (ion source: 230°C, and quadrupole: 150°C). Compounds were identified by matching their spectra to spectra from the NIST 02 and Wiley mass spectral libraries. The identity of the following terpenes was confirmed by comparison of spectra with those of reference standard compounds purchased from Sigma-Aldrich: α-pinene, β-pinene, δ-3-carene, camphene, β-myrcene, limonene, γ-terpinene, terpinolene, eucalyptol, verbenone, α-cedrene, caryophyllene, and (*E*)-β-farnesene.

Quantification of compounds in SuperQ samples was based on GC-FID peak area compared to the peak area of nonyl acetate as the internal standard. Peak area values were corrected according to the relative response factors. For components with a purchased reference standard available (see above), the response factors relative to that of nonyl acetate were determined based on the slope value of the respective calibration curves. Terpenes identified based on their mass spectrum had the relative response factor of one of the reference compounds assigned depending on which compound class they belong to (aliphatic, monocyclic, or bicyclic monoterpene hydrocarbon, aliphatic or cyclic sesquiterpene hydrocarbon, monoterpene alcohol, or ketone). Since limonene and β-phellandrene were not separable on the Equity-5 column, they were quantified as one compound.

Volatile Emission Calculations The hourly volatile emission rate of the whole trunk section of the trees through the bark was estimated using the calculated amount of volatiles collected in 30 min based on the GC data. The collection covered a surface area of about 0.365 m^2. Considering the trunk of an average pine tree to be a truncated cone with a height of about 12 m, a base diameter of about 25 cm, and the top diameter of about 2 cm, the surface area of such a trunk is about 5.244 m^2. Thus, we used a factor of 14 for the surface area and a factor of 2 for the time period resulting in a combined factor of 28. This factor was multiplied by the measured emission rate to yield total emission rates per tree-trunk per hour.

Statistical Analyses Data were analyzed starting from week 4 since no *Sirex* was captured on the traps prior to that week. Outlier volatile emission rate values were not included for one of the two reasons: 1) The tree was substantially damaged during trap setup causing elevated volatile emission rates at the beginning of the season, or 2) there was resin contamination in the chamber (which usually happened towards the end of the season).

Total trap captures and the chemical composition data were analyzed for differences between treatment groups using a mixed model (Littell et al. 2006) with treatment as fixed effect, and site and site*treatment interaction as random factors. In the analysis of weekly trap captures and weekly emission rates, week and treatment*week interaction were also added as fixed effects with site*week and site*treatment*week interactions as random factors. Tree-to-tree variation was accounted for in the residual variances. Competing models (separate residual variances vs. common residual variances) were compared using Akaike's Information Criterion (AIC), and the model with the lowest AIC was selected. Separate residual variances were used for each treatment when necessary to attain normality and homogeneity of variances. All trapping and volatile emission data were square-root transformed, and the chemical composition data were square-root transformed when necessary to attain normality. For pairwise comparison of the Least Square Means (LS Means), Tukey multiple-range test was performed using the pdmix800 SAS macro (Saxton 1998). No statistical tests were performed on the emergence data due to extremely low sample sizes.

Results

Needles began to turn brown in both pine species as a symptom of stress by the 4th week after stem injection with the herbicide Dicamba. No such effect was observed with the control pine trees. The whole canopy of herbicide-treated (HT) Scots pines (*Pinus sylvestris*) turned brown by the 7th week whereas it happened to white pines (*P. strobus)* only by the 9th week.

Trapping Only female *Sirex noctilio* were captured in our study: a total number of 120 at all three sites combined. As

in 2008, the majority of females were caught by HT Scots pines (92), about two-thirds of which were caught by the high-carene chemotype (64). There was a significant difference in the total trap captures between treatment groups (4.93, 0.016, *F*, *P*). The numbers of *S. noctilio* females caught by HT trees were higher than the numbers caught by the respective controls, and the difference was significant for the high-carene chemotype (P=0.033) (Fig. 1). The difference between HT and control was not significant in low-carene Scots pine or white pine. Herbicide-treated trees of both Scots pine chemotypes captured more females than HT white pine, and the difference was significant between HT high-carene Scots pine and HT white pine (P=0.037).

The peak in trap captures was in the second half of July, 6 to 7 week after the herbicide treatment, about a week later than in the previous year. The statistics showed a weak overall week effect (Table 2), with week 7 being statistically different from week 4, and marginally statistically different from week 10 (P=0.036 and 0.081, respectively). The overall treatment effect was highly significant (Table 2), but there was no treatment*week interaction (1.30, 0.138, *F*, *P*). Weekly trap captures of herbicide-treated trap trees peaked 6 to 7 weeks after stem injection for the high-carene Scots pine and at week 5 to 6 for the low-carene chemotype (back-transformed LS Means are presented in Fig. 2a). White pine trap captures increased slightly in the middle of the season but stayed at a low level. The weekly trap captures differed significantly between treatment groups at week 6 and 7 (6.56, <0.001 and 6.40, <0.001, *F*, *P*, respectively). The trend among herbicide-treated groups in that period was such that high-carene Scots pine caught more wasps than low-carene Scots pine, which caught more wasps than white pine. However, significant difference was found only between HT high-carene Scots pine and HT white pine and only at week 6 (P=0.021).

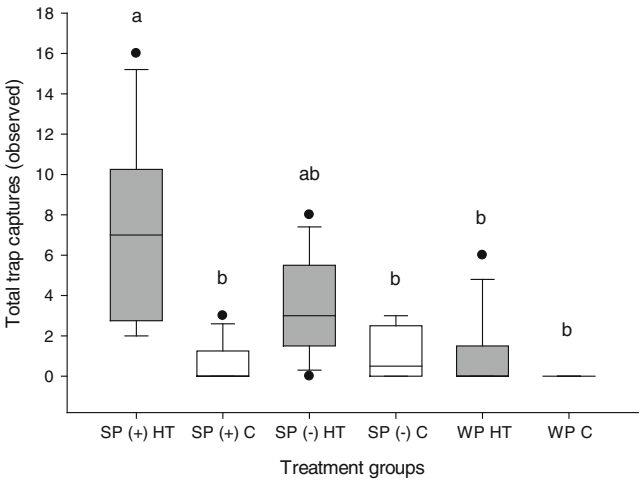

Fig. 1 Total number (observed data) of *Sirex noctilio* females captured by trap trees of the different treatment groups: SP (+) HT: herbicide-treated high-carene Scots pine, SP (+) C: control high-carene Scots pine, SP (−) HT: herbicide-treated low-carene Scots pine, SP (−) C: control low-carene Scots pine, WP HT: herbicide-treated white pine, and WP C: control white pine. Sample size for low-carene Scots pine is N=8, otherwise N=9. A mixed model was used to analyze the square-root transformed data (see Methods) and a significant treatment effect (F=4.93, P=0.016) was found. For pairwise comparison of back-transformed LS Means Tukey multiple-range test was used at α=0.05 significance level. Treatment groups not connected by the same letter are significantly different

Table 2 Tests of fixed effects in the mixed model for the trap capture and volatile emission rate data

Effect	Trap captures		Volatile emission rates	
	F value	*P* value	*F* value	*P* value
Treatment	9.65	<0.001	14.09	<0.001
Week	2.34	0.033	4.46	0.004
Treatment*week	1.30	0.138	1.19	0.289

Sites were different in the total number of *S. noctilio* females captured: 77, 28, and 15, and the site with the lowest number had a later peak in trap captures, at week 9, compared to the other sites (at week 6 to 7). These findings were reflected in the non-zero values of the covariance parameter estimates for site and site*week interaction in the statistical model.

Analysis of Volatiles The overall peak in emission rates was at week 5 to 6 after which the rates slowly declined with week 5 being significantly different from week 9 and 11 (Table 3). The back-transformed LS Means of the weekly volatile emission rates for each treatment group are presented in Fig. 2b. A strong treatment effect was observed, as in the trapping data, and also a strong week effect, but the treatment*week interaction was not significant (Table 2). While herbicide-treated Scots pines emitted significantly higher amounts of volatiles per hour than the respective control trees, stem injection did not cause a significant increase in the volatile emission rates of white pines (Table 3). When comparing the different herbicide-injected groups, Scots pines emitted significantly higher amounts per hour than white pine, but there was no significant difference in the emission rates of the two chemotypes of Scots pine (Table 3). As opposed to the trapping data, there were no site differences in the volatile emission rates, indicated by an estimated zero covariance for site as a random effect in the mixed model. However, the tree-to-tree variation was high, much higher than any other random effects (site*treatment, site*week, or site*treatment*week interactions).

In summary, when comparing quantities of volatiles emitted by high-carene Scots pine and white pine, higher trap captures paralleled higher emission rates. Nevertheless, while having comparable volatile emission rates to that of HT high-carene Scots pines, the low-carene Scots pines captured fewer *S. noctilio* females in comparison.

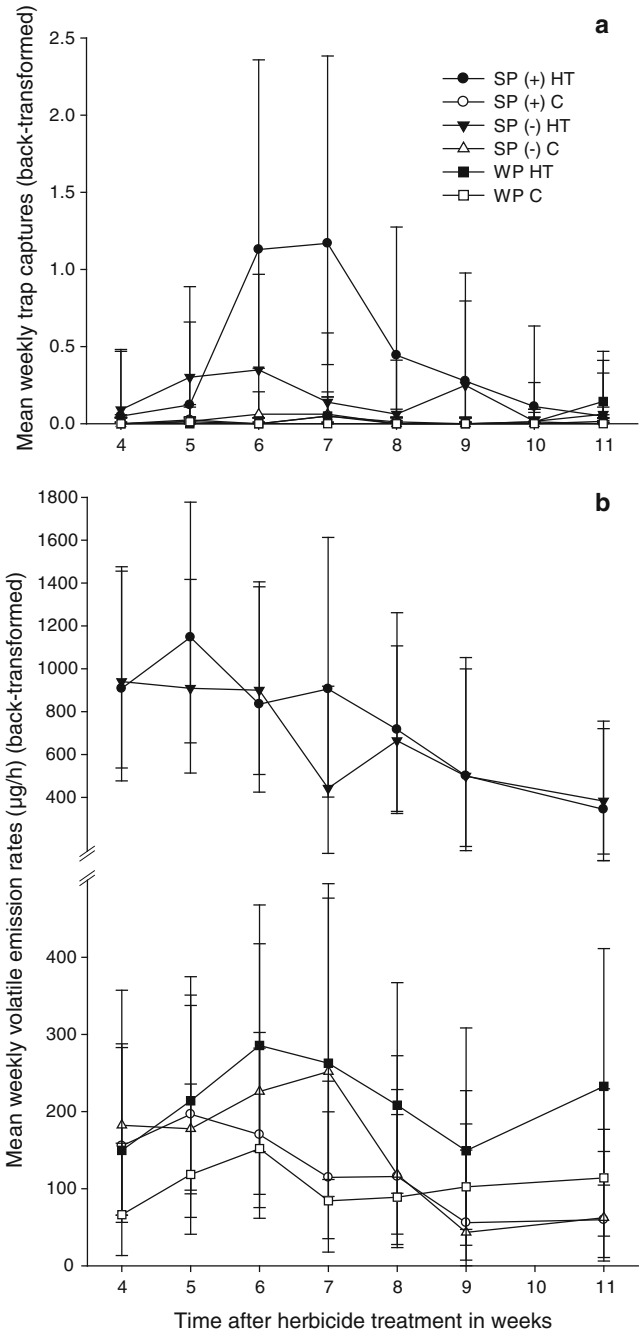

Figure 2 Mean weekly trap captures (back-transformed LS Means) of *Sirex noctilio* females by treatment groups (N=8–9), **a**, and the corresponding mean weekly volatile emission rates (back-transformed LS Means) (N=4–9), **b** *Error bars* represent the lower and upper 95% confidence intervals. A mixed model was used to analyze the square-root transformed data; for detailed results see Table 2 and 3

Qualitative differences were found in the volatile profiles of the stem injected treatment groups due to differential emission of some of the terpenes (Table 4). Percent composition of selected monoterpenes was compared at week 6 when there was a peak in trap captures with a significant treatment effect (6.56, <0.001, F, P). The two monoterpenes α- and β-pinene were major components in the volatile blend of all herbicide-treated groups. In high-carene Scots pine and white pine samples, δ-3-carene was a major component, too, although it was significantly less abundant in HT white pine compared to HT high-carene Scots pine (P<0.001). The sum of these three monoterpenes accounted for about 90% of the volatiles emitted in all herbicide-treated groups. Herbicide injection induced relatively higher emission rates of α-pinene in low-carene Scots pine only (P=0.006) (Table 4). The relative emission rates of β-pinene and δ-3-carene did not change significantly upon herbicide treatment for either species or chemotype. Some of the minor components, such as α-thujene, γ-terpinene, and terpinolene were present in significantly higher amounts in HT high-carene Scots pine compared to the other HT groups, and thus were positively linked to emission levels of δ-3-carene (Table 4). In addition, sabinene was significantly more abundant in high-carene Scots pine than in the low-carene chemotype and somewhat more abundant than in white pine. The relative emission rates of these minor components increased in high-carene Scots pine upon herbicide treatment. Other components, however, such as tricyclene, camphene, and limonene together with β-phellandrene, were emitted at a lower rate by HT Scots pines compared to HT white pine (trend only). The enantiomeric ratio (mean±SE) of α-pinene, (*R*)-/(*S*)-, in HT high-carene Scots pine tended to be higher (4.4±1.2) than in the other two HT groups (1.7±0.3 and 1.4±0.2 for low-carene Scots pine and white pine, respectively), however, the difference was not significant. As a trend, herbicide treatment resulted in higher (*R*)-/(*S*)- ratios in HT high-carene compared to the control. Oxygenated monoterpenes occurred in traces in most of the samples, therefore, only the total amounts are listed in Table 4. The relative emission rate of these compounds was lower in HT Scots pines than in HT white pines, however not significantly. Sesquiterpenes could not be quantified due to the extremely small amounts detected in the samples. Thus, this compound group is not discussed in the paper.

Sirex noctilio Emergence Control trees of low-carene Scots pine and white pine had zero *S. noctilio* emergence from the sampled material, whereas the high-carene controls provided a few emerging woodwasps (5.67±5.67). In HT trees, more *S. noctilio* emerged from the low-carene Scots pines (13.00±5.15) than from the high-carene Scots pines (1.67±5.15) and white pines (4.33±5.15).

Discussion

Little is known about the mechanism by which the woodwasp *Sirex noctilio* finds a suitable pine host for oviposition. Regardless of the possible mode of action (attractant or

Table 3 Separation of treatment groups and weeks by volatile emission rates (μg/h)

High-carene Scots pine		Low-carene Scots pine		White pine		
HT	Control	HT	Control	HT	Control	
742 (510, 1017) [a]	119 (49, 217) [b]	658 (446, 910) [a]	141 (60, 255) [b]	212 (113, 341) [b]	102 (39, 195) [b]	
Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 11
319 (231, 420) [abc]	380 (283, 490) [a]	375 (279, 485) [ab]	295 (196, 415) [abc]	267 (186, 363) [abc]	183 (106, 280) [bc]	176 (112, 255) [c]

HT herbicide-treated, Control: untreated

Back-transformed LS Means are presented with the lower and upper 95% confidence intervals in parentheses. Treatment groups or weeks not connected by the same letter are significantly different (Tukey multiple-range test, α=0.05)

arrestant) we have been looking for compounds emitted by stressed pines that may govern the host selection of the wasp. We analyzed volatiles from Scots pine (*P. sylvestris*) and white pine (*P. strobus)*. Our behavioral assay was a field trapping study conducted in NY State in 2009. We demonstrated that high-carene Scots pines captured more females than the low-carene Scots pine chemotype or than white pines at the three mixed sites where trees had been stressed by stem injection with the herbicide Dicamba 4 week before the anticipated peak flight. Moreover, Zylstra et al. (2010) reported higher numbers of female woodwasps attracted to Scots pine than to red pine (*P. resinosa*), in a similar experimental setup. Thus, Scots pine, and especially the high-carene chemotype, is worthy of study as a potential source of kairomones that might be effective lure components.

We note that trap captures provide no information on differences in egg density or larval performance, i.e., the suitability of the tree for the development of the insect (Morgan and Stewart 1972). Therefore, experiments that test host preference by keeping females in a confined space with wood material might deliver a different ranking of pine species. In fact, we did not find a big difference in the emergence between the two host species, despite lower trap captures in white pine. This suggests that while volatiles draw woodwasps to the host, other factors, such as resin pressure and moisture content of the trees, affect the choice to oviposit and the success of development therein.

The total number of *S. noctilio* captured at each site varied in a wide range (15–77). The sites are relatively small stands on private properties in the Syracuse, NY area (separated by

Table 4 Mean percent compositions of selected monoterpenes (observed data) in samples collected 6 weeks after stem injection

	High-carene Scots pine		Low-carene Scots pine		White pine		Treatment effect[a]
	HT[b]	Control	HT	Control	HT	Control	*F*/*P*
α-Pinene	52.9±5.8[b]	54.0±2.7 [b]	80.9±3.8[a]	64.4±3.0[b]	56.4±2.7[b]	55.0±2.3[b]	7.67/<0.001
(*R*)-/(*S*)-	4.4±1.2[a]	2.3±0.04[a]	1.7±0.3[a]	1.7±0.2[a]	1.4±0.2[a]	1.6±0.2[a]	2.79/0.050
β-Pinene	13.6±4.3[a]	15.3±2.3[a]	12.6±3.8[a]	20.7±2.4[a]	26.4±3.5[a]	25.4±2.7[a]	3.27/0.030
δ-3-Carene	25.8±3.4[a]	17.8±2.7[a]	0.5±0.2[c]	5.0±2.0[bc]	5.1±1.6[b]	6.7±1.8[b]	20.12/<0.001
Tricyclene	0.17±0.02[b]	0.29±0.05[ab]	0.21±0.02[b]	0.34±0.05[ab]	0.42±0.06[a]	0.42±0.06[a]	7.61/<0.001
Thujene	0.08±0.01[a]	0.02±0.01[b]	0.00±0.00[b]	0.00±0.00[b]	0.01±0.01[b]	0.01±0.01[b]	9.04/<0.001
Camphene	0.7±0.1[b]	1.1±0.1[a]	1.2±0.1[a]	1.4±0.1[a]	2.8±0.6[a]	2.3±0.4[a]	7.83/0.001
Sabinene	0.62±0.11[a]	0.10±0.06[b]	0.02±0.01[b]	0.06±0.06[b]	0.37±0.17[ab]	0.40±0.27[ab]	7.37/0.002
β-Myrcene	1.2±0.1[a]	0.8±0.1[a]	0.9±0.1[a]	0.6±0.1[a]	1.2±0.3[a]	1.0±0.2[a]	1.61/0.348
Lim/β-phell[c]	2.0±0.2[b]	3.1±0.3[a]	2.4±0.2[ab]	3.3±0.3[a]	3.2±0.2[a]	3.3±0.3[a]	4.12/0.004
γ-Terpinene	0.18±0.02[a]	0.09±0.04[ab]	0.01±0.00[b]	0.00±0.00[b]	0.02±0.01[b]	0.01±0.01[b]	24.89/<0.001
Terpinolene	1.27±0.18[a]	0.58±0.20[ab]	0.17±0.03[b]	0.14±0.06[b]	0.27±0.05[b]	0.21±0.08[b]	6.99/0.003
OMT[d]	0.3±0.1[a]	1.0±0.6[a]	0.8±0.5[a]	0.3±0.2[a]	0.9±0.4[a]	0.8±0.4[a]	0.93/0.500

[a] A mixed model was used to analyze the data (see Methods). For pairwise comparison of LS Means Tukey multiple-range test was used at α=0.05 significance level. Levels for a given compound not connected by the same letter are significantly different. Data analysis was performed on square-root transformed data for β-pinene, sabinene, γ-terpinene, and oxygenated monoterpenes (OMT)

[b] *HT* herbicide-treated, Control: untreated

[c] *Lim/β-phell* limonene and β-phellandrene were quantified together as their GC peaks were not resolved on the Equity-5 column

[d] *OMT* sum of oxygenated monoterpenes

up to 50 km), and the population density of *S. noctilio* varies significantly among them. Even though they were selected based on typical signs of attack by *S. noctilio*, such as resin beads, brown crowns, and emergence holes, the density of the wasp population in a particular pine stand is hard to predict before flight. An added challenge was to find enough Scots pines of both chemotypes at sites that also had white pines.

We examined the relationship between trap captures and the volatile emission rates of herbicide-treated trees. While herbicide-treated high-carene Scots pines captured significantly more female woodwasps and emitted volatiles at a significantly higher rate than herbicide-treated white pines, there was no significant difference in the volatile emission rates between the two chemotypes of Scots pine, yet, the high-carene chemotype captured more woodwasps. Moreover, in a study conducted in 2006, we found that while herbicide-treated red pines emitted similar amounts of volatiles as Scots pines the trap captures were lower (unpublished data of the authors). Thus, quantitative differences in the volatile emission rates only partly explain the differences seen in trap captures.

We found qualitative differences among the volatile blends of the two chemotypes of Scots pine and white pine. It is not only δ-3-carene that distinguishes the volatile blends of high- and low-carene chemotypes (Thoss et al. 2007), but some minor monoterpenes, as well, namely thujene, sabinene, γ-terpinene, and terpinolene. As these components had elevated levels in high-carene Scots pine compared to the other groups, they are good target compounds for a behavioral assay. Although they did not elicit high electrophysiological responses when tested individually (Simpson 1976), these compounds still might be important components of an attractive blend.

The ratios of the (*R*)-(+)- and (*S*)-(−)-enantiomers of α-pinene were higher in the high-carene samples compared to the low-carene and white pine samples collected 6 week after stem injection, although not significantly. According to Sjödin et al. (1993) there was no significant treatment effect on the enantiomeric ratios of chiral components when Scots pine trees were mechanically wounded. Neither did sawfly (*Diprion* pini) oviposition on needles of *P. sylvestris* affect the enantiomeric ratios of needle volatiles (Mumm et al. 2003). Nevertheless, elevated (*R*)-(+)-α-pinene emission was found in artificially wounded *P. massoniana* trees that attracted the pine sawyer beetle *Monochamus alternatus* (Fan et al. 2007). The relevance of changes in enantiomeric ratios needs to be tested in bioassays.

It is not clear whether the compounds/blends emitted by 'attractive' trees act, in fact, as attractants from a long distance or rather, as arrestants after an "appropriate landing" (Finch and Collier 2000). Madden (1988) reported on the arrestment activity of α- and β-pinene in flight mill olfactometers, however, no data were presented. Visual cues also need to be considered when developing a trapping method (Reddy et al. 2005 and the references therein). In the case of the mountain pine beetle, *Dendroctonus ponderosae*, Campbell and Borden (2006) found that olfactory and visual cues are integrated at close-range to the host.

When trying to explain why a certain host species is preferred, we often neglect the lack of non-host characteristics that might contribute to the 'attractiveness' of the host. In a comparative study of preferred (*P. sylvestris* and *P. nigra*) and non-preferred (*P. pinea*) host species of the pine sawfly, *Neodiprion sertifer*, the monoterpene limonene induced avoidance when present in higher relative amounts (Martini et al. 2010). In *S. noctilio*, the oxygenated monoterpene components of *P. radiata* resin are thought to be repellents, or compounds that mask the attractiveness of the monoterpene hydrocarbons (Taylor 1981), although never tested in a bioassay. One of the oxygenated monoterpene components of *P. radiata* resin, camphor, was detected in our samples as well. We found relatively low amounts of oxygenated monoterpenes in herbicide-treated Scots pines compared to the other herbicide-treated groups, although the very low amounts made it impossible to individually quantify these components.

The volatile collection method used in our study, i.e., collecting airborne compounds on a sorbent, is especially suitable for field collections when multiple emitters have to be sampled, and when there are no opportunities to analyze samples immediately after collection. Eluting the sorbent with a solvent allows the researcher to run multiple tests (chemical and behavioral) with the sample and to perform quantification of the compounds. Nevertheless, the detection of low-molecular weight compounds that elute with the solvent from the GC column is not possible using this method. It is likely that we missed volatile components relevant to the host-seeking behavior of *S. noctilio*. Although solvent-free methods such as thermodesorption (Helsper et al. 2006) or collection on solid-phase microextraction (SPME) fibers (Martini et al. 2010) provide only one-time analysis, they are useful techniques in the exploration of volatile chemical cues. Testing compounds more abundant in the volatile profile of attractive pine species, such as δ-3-carene, thujene, sabinene, γ-terpinene, and terpinolene in high-carene Scots pine, with electrophysiological methods and in behavioral assays will be a focal point of our investigations in the future. Such research is valuable because it could lead to the identification of a successful artificial lure that will improve detection efficacy of insect pests for forest managers working to conserve and protect ecologically significant habitats and economic commodities.

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RAPID COMMUNICATION

Plant Volatiles Enhance Behavioral Responses of Grapevine Moth Males, *Lobesia botrana* to Sex Pheromone

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Abstract Plant volatiles play an important role in the lives of phytophagous insects, by guiding them to oviposition, feeding and mating sites. We tested the effects of different host-plant volatiles on attraction of *Lobesia botrana* males to the female-produced sex pheromone, in a wind tunnel. Addition of volatile emissions from grapevines or individual plant volatiles to pheromone increased the behavioral responses of *L. botrana* males over those to pheromone alone. At a low release rate (under-dosed) of pheromone, addition of (*E*)-β-caryophyllene, (*Z*)-3-hexenyl acetate, 1-hexanol, or 1-octen-3-ol increased all behavioral responses, from activation to pheromone source contact, while addition of (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-β-farnesene, (*Z*)-3-hexenol, or methyl salicylate affected only the initial behavioral responses. Dose–response experiments suggested an optimal release ratio of 1:1000 (sex pheromone: host plant volatile). Our results highlight the role of plant volatiles in the sensory ecology of *L. botrana*.

Keywords Pheromone response · Kairomone · Wind tunnel · *Vitis vinifera* · European grapevine moth · Tortricidae · Lepidoptera

Introduction

Host-plant volatiles can enhance both pheromone production and behavioral responses to pheromones in phytophagous insects (reviewed by Landolt and Phillips, 1997). Host-plant attractants have been extensively studied for *Lobesia botrana* (Lepidoptera: Tortricidae) females (Tasin et al., 2006, 2007; Anfora et al., 2009), but work focusing on the effects of plant volatiles on the behavior of *L. botrana* males is sparse. *L. botrana* uses a female-produced sex pheromone for mate finding. During the scotophase, male moths compete heavily for females, with males arriving at the calling female first most likely to achieve mating success. We hypothesized that host-plant volatiles could signal rendezvous sites by enhancing male behavioral responses to pheromone in *L. botrana*. For this, we studied the effects of host-plant volatiles on the attractiveness of the sex pheromone, and explored dose–response characteristics of behaviorally active plant volatiles.

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Methods and Materials

Insects

Grapevine moth larvae were reared on artificial diet under a 16 L:8D photoperiod, 65% RH and 25°C during photophase, and 85% RH and 18°C during scotophase, as described in von Arx et al. (2011).

Test Compounds

Pheromone components (isomeric purities) used were: (*E,Z*)-7,9-dodecadienyl acetate (>97%), (*E,Z*)-7,9-dodecadien-1-ol (>94%), and (*Z*)-9-dodecenyl acetate (99.9%, Plant Research International, Wageningen, Netherlands), admixed at a ratio of 100:20:5 (Arn et al., 1988). Plant compounds tested were those shown to elicit antennal receptor cell responses in *L. botrana* males, or that had been identified as key semiochemicals in behavioral assays with *L. botrana* females (Tasin et al., 2007, von Arx et al., 2011): 1-hexanol,

(*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, (*R*)-(+)-limonene, (*E*)-β-caryophyllene, (+)-terpinen-4-ol, and methyl salicylate (all >94%, Sigma-Aldrich, Buchs, Switzerland), 1-octen-3-ol (>97%, Merck, Munich, Germany), (*E*)-β-farnesene (>90%, Bedoukian Research, Danbury, CT, USA) and (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT, ~94%, Givaudan, Dübendorf, Switzerland). Ethanol (pro Analysis, Merck, Munich, Germany) was used as solvent.

Wind Tunnel

Experiments were carried out in a wind tunnel, equipped with a piezo sprayer (see von Arx et al., 2011 for the setup, dimensions and experimental conditions). Dose–response trials were performed with the ternary pheromone blend (0.01–100,000 pg.min^{-1}; data not shown) to establish optimal (1.0 pg.min^{-1}) and under-dosed pheromone release rates (0.1 pg.min^{-1}). In our first trial, pheromone (1.0 pg.min^{-1}) was presented in a grapevine volatile background (8 vine plants, *Vitis vinifera* cv. Solaris, placed between the charcoal filters and the perforated metal grid at the upwind end of the wind tunnel; plant height: 45±5 cm, wet mass of leaves: 20±2 g/plant). Then, we tested responses of males to single plant chemicals at a suboptimal dose (0.1 pg.min^{-1}) of pheromone. The suboptimal dose was used because behavioral responses of male *L. botrana*, in terms of takeoff (78%) and upwind flight (59%), were already high to the optimal pheromone rate (1.0 pg.min^{-1}) in the absence of plant volatiles. Single compounds were tested first at 100 pg.min^{-1}. Then, the 6 compounds that showed the strongest effect when added to pheromone were tested at additional release rates of 1.0 pg.min^{-1} and 10,000 pg.min^{-1}. Three-day-old males were presented individually to the odor source and scored for: (1) activation, (2) takeoff, (3) upwind flight, (4) passing the midline of the wind tunnel, and (5) odor source contact.

Statistical Analysis

Treatments were compared by fitting a generalized linear model (GLM) with a logit link function (logistic regression) to the behavioral responses, assumed to be binomially distributed (R 2.11.1). Analysis of deviance, based on the asymptotic $\chi 2$ distribution, was used to test whether flight responses were dependent on odor source ($P<0.05$), with treatment differences separated by Tukey- or Dunnett-contrasts.

Results

Grapevine volatile emissions increased the attractiveness of the optimal pheromone dose, with more males engaging in upwind flight and contacting the odor source to the combined odors than to the optimal pheromone dose alone (Table 1). The ten host-plant volatiles, tested individually at a release rate of 100 pg.min^{-1}, influenced flight behavior of grapevine moth males to under-dosed pheromone as follows: (1) (*E*)-β-caryophyllene, (*Z*)-3-hexenyl acetate, 1-hexanol, or 1-octen-3-ol increased the number of males undertaking all behavioral elements, (2) DMNT, (*E*)-β-farnesene, (*Z*)-3-hexenol, or methyl salicylate enhanced only initial behavioral elements, and (3) (*R*)-(+)-limonene or (+)-terpinen-4-ol had no effect on the attractiveness of the pheromone blend.

Host-plant volatiles also increased the attractiveness of under-dosed pheromone when tested at 1.0 and 10,000 pg.min^{-1} (Table 1). At 10,000 pg.min^{-1}, 1-hexanol or 1-octen-3-ol increased activation and take off rates, while (*E*)-β-caryophyllene enhanced the full behavioral repertoire to pheromone. At 1.0 pg.min, 1-octen-3-ol enhanced activation, while (*E*)-β-caryophyllene increased both activation and takeoff. The amount of plant volatile released influenced male behavior, in general, with the number of moths displaying a given behavioral element higher at 100 pg.min than at 1.0 or 10,000 pg.min.

Discussion

We provide evidence that the perception of plant volatiles facilitates mate finding in *L. botrana*; plant volatiles admixed in a 1000:1 ratio with under-dosed pheromone attracted as many males as the optimal pheromone concentration. Thus, responses to low pheromone concentrations are increased when host-plant volatiles are perceived simultaneously. This simultaneous perception likely aids males to locate females and increases their chances of mating. The compounds, (*E*)-β-caryophyllene, (*Z*)-3-hexenyl acetate, (*Z*)-3-hexenol, methyl salicylate, and (*E*)-β-farnesene, have been found previously to enhance attraction of *Eupoecilia ambiguella* and *Cydia pomonella* to their respective sex pheromones (Yang et al., 2004; Schmidt-Buesser et al., 2009). Furthermore, 1-hexanol, (*E*)-β-caryophyllene, (*Z*)-3-hexenyl acetate, or 1-octen-3-ol attract grapevine moth males on their own when used at similar release rates as in the current study (von Arx et al., 2011). The lack of response, reported by Masante-Roca et al. (2007), of *L. botrana* males to grapevines could be due to variables associated with the plant material tested (e.g., grape variety, amount of plant material, cut vs. undamaged plants and the release rate of plant volatiles).

The compounds tested in our study are not unique to grapevine, raising the question of specificity of these signals. Although we tested plant compounds individually, one might expect multi-component blends to enhance attractiveness of under-dosed pheromone to a greater extent, based on our

Table 1 Effects of plant volatiles on the attractiveness of the female sex pheromone to *Lobesia botrana* males

		Behavioral responses[a]					
Treatments[b]	Release rate[c] (pg.min^{-1})	Activation	Take off	Upwind	Midline	Contact	*N*
$P_{1.0}$ (optimal)+grapevine	not known	89^{***}	88^{***}	$75^{a\ ***}$	73^{***}	$71^{a\ ***}$	85
$P_{1.0}$	–	80^{***}	78^{***}	$59^{b\ **}$	59^{**}	$49^{b\ *}$	85
$P_{0.1}$+1-hexanol	1	61^{b}	58^{b}	40^{b}	40^{b}	35^{b}	80
	100	$78^{a\ **}$	$73^{a\ **}$	$61^{a\ **}$	$61^{a\ **}$	$55^{a\ *}$	80
	10,000	$77^{a\ ***}$	$59^{ab\ *}$	39^{b}	38^{b}	33^{b}	120
$P_{0.1}$+(*E*)-β-caryophyllene	1	$79^{a\ ***}$	$65^{a\ *}$	36^{b}	34^{b}	26^{b}	80
	100	$76^{a\ ***}$	$73^{a\ ***}$	$56^{a\ **}$	$56^{a\ **}$	$56^{a\ **}$	80
	10,000	$82^{a\ ***}$	$71^{a\ ***}$	$53^{a\ *}$	$52^{a\ *}$	$48^{a\ *}$	79
$P_{0.1}$+(*Z*)-3-hexenyl acetate	1	61^{b}	55^{b}	34^{b}	33^{b}	31^{b}	80
	100	$85^{a\ ***}$	$74^{a\ ***}$	$58^{a\ **}$	$53^{a\ *}$	$51^{a\ *}$	80
	10,000	63^{b}	58^{b}	34^{b}	34^{b}	25^{b}	80
$P_{0.1}$+1-octen-3-ol	1	$71^{b\ **}$	60^{b}	43^{a}	40^{a}	36^{ab}	80
	100	$89^{a\ ***}$	$79^{a\ ***}$	$56^{a\ **}$	$54^{a\ *}$	$50^{a\ *}$	80
	10,000	$66^{b\ *}$	$61^{b\ *}$	41^{a}	40^{a}	34^{b}	80
$P_{0.1}$+(*Z*)-3-hexenol	1	59^{b}	48^{b}	34^{b}	30^{b}	29^{b}	80
	100	$84^{a\ ***}$	$71^{a\ ***}$	$50^{a\ *}$	$50^{a\ *}$	44^{a}	80
	10,000	54^{b}	50^{b}	36^{ab}	36^{b}	30^{ab}	80
$P_{0.1}$+methyl salicylate	1	61^{b}	46^{b}	33^{b}	27^{b}	23^{b}	70
	100	$78^{a\ ***}$	$74^{a\ ***}$	49^{a}	49^{a}	46^{a}	80
	10,000	55^{b}	49^{b}	36^{ab}	34^{ab}	34^{ab}	80
$P_{0.1}$+(*E*)-β-farnesene	100	74^{**}	66^{**}	49	45	43	80
$P_{0.1}$+DMNT	100	70^{**}	59	38	34	31	80
$P_{0.1}$+(+)-terpinen-4-ol	100	60	45	33	33	33	40
$P_{0.1}$+(*R*)-(+)-limonene	100	60	45	30	28	20	40
$P_{0.1}$ (under-dosed)	–	49	45	34	34	33	80
EtOH (blank)	–	8	6	1	1	1	90

[a] Percentage of *L. botrana* males showing a particular behavioral response. *N* is the number of males tested for each treatment. Different letters assigned within a response indicate differences: (1) between responses to the two treatments with the optimal pheromone dose, and (2) among responses to the three release rates within a pheromone+plant volatile treatment (GLM, $P<0.05$). Asterisks among a behavioral response indicate differences between the under-dosed pheromone mixture ($P_{0.1}$) and the other treatments (GLM, $P\leq0.05$*, $P\leq0.01$**, $P\leq0.001$***; the ethanol control (blank) was not included in the analysis)

[b] Sex pheromone treatments consisted of a 3-component blend of *L. botrana* sex pheromone components (see text). The $P_{1.0}$ optimal dose (1.0 pg.min^{-1}) was presented in a background of grapevine plants and the $P_{0.1}$ under-dosed level (0.1 pg.min^{-1}) was presented in combination with the different release rates of individual plant compounds

[c] Release rate of plant volatile chemicals

finding that grapevine emissions had additive effects on moth attraction when added to the optimal pheromone dose (Table 1), and from experiments in which multi-component blends were more attractive to grapevine moths than simpler blends or single components (Tasin et al., 2007; von Arx et al., 2011). Because of the high responses of *L. botrana* males to the optimal pheromone dose, we explored the effects of single host-plant volatiles on pheromone attraction at an under-dosed level. Refining host-plant volatile mixtures, to add to the pheromone to improve attraction, could be the subject of future work.

With the exception of (*E*)-β-caryophyllene, a general pattern emerged from our dose–response experiment, of an optimal plant volatile release rate of 100 pg.min^{-1} when using the under-dosed pheromone level. This fits well with release rates of compounds from grapevine inflorescences and berries (Tasin et al., 2006; Anfora et al., 2009). (*E*)-β-Caryophyllene increased attractiveness of pheromone over a wider range of release rates than the other compounds tested, indicating that grapevine moth males might be able to accommodate substantial variation in the amounts of some compounds, as shown for DMNT in the moth *Paralobesia viteana* (Cha et al., 2011).

Our results indicate that some plant compounds attract both sexes of *L. botrana*. The three terpenes, (*E*)-β-caryophyllene, DMNT, and (*E*)-β-farnesene, as well as methyl

salicylate and 1-octen-3-ol, were present in a headspace collection of grapevine that elicited upwind flight of mated females (Tasin et al., 2006). In a subsequent subtractive bioassay of this headspace collection, (*E*)-β-caryophyllene, DMNT, and (*E*)-β-farnesene were identified as essential components for eliciting upwind flight in mated *L. botrana* females (Tasin et al., 2007).

In summary, we demonstrated that responses of *L. botrana* males to sex pheromone are improved in the presence of plant compounds, and we provide evidence for the hypothesis that grapevine moth males use volatile host-plant cues in mate-finding. Future field experiments will test whether addition of plant volatiles to sex pheromone improve the efficiency of mating disruption techniques, perhaps by increasing the active range of dispensers.

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RAPID COMMUNICATION

Volatile Dose and Exposure Time Impact Perception in Neighboring Plants

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Abstract Volatiles emitted from stressed plants can induce resistance in healthy neighbors. It remains unknown, however, how plants perceive volatiles and convert them into internal signals. We exposed lima bean (*Phaseolus lunatus* L.) to different concentrations of either of two volatiles, nonanal and methyl salicylate (MeSA), over 6 or 24 h. Plant resistance to the bacterial pathogen, *Pseudomonas syringae*, was increased significantly after exposure to a headspace with two concentrations of nonanal for 6 h, and the same pattern emerged after an exposure over 24 h. By contrast, exposure to a low concentration of MeSA over 6 h did not significantly reduce bacterial infections, whereas exposure to the same concentration over 24 h significantly enhanced resistance. The dose–response relation that was apparent after 6 h of MeSA exposure disappeared in the 24 h treatment, in which the three tested concentrations caused indistinguishable, high levels of resistance to *P. syringae*. A low concentration of a potentially resistance-enhancing volatile sufficed to cause resistance to pathogens in the receiver plant only after long exposure time. Plant-plant signaling appears to involve the accumulation of volatiles in the receiver.

Keywords Induced resistance · Volatile organic compounds · VOCs · Plant pathogenic bacterium · Plant-plant signaling

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Introduction

Healthy plants can enhance their resistance to herbivores, pathogens, or abiotic stress when being exposed to volatile organic compounds (VOCs) that are emitted from stressed plant neighbors. This plant-plant signaling has been described for multiple species comprising monocots and dicots (Heil and Karban, 2010). Field studies conducted with alder (*Alnus glutinosa*), wild tobacco (*Nicotiana attenuata*) exposed to clipped sagebrush (*Artemisia tridentata*), or lima bean (*Phaseolus lunatus* L.), demonstrated that plant-plant signaling functions over short distances of 30 cm up to 1 m (Dolch and Tscharntke, 2000; Karban et al., 2003; Heil and Adame-Álvarez, 2010). This distance can decrease in the presence of ozone (Blande et al., 2010). All these observations indicate that the underlying signals are rapidly diluted or degraded to inactive concentrations. It remains unknown, however, how long a plant needs to be exposed to which concentration of a volatile, in order to exhibit a detectable response. Here, we aimed at determining the concentration and minimum time of exposure of lima bean to either of two volatiles, nonanal and methyl salicylate (MeSA). Both compounds enhance resistance in lima bean to the bacterial pathogen, *Pseudomonas syringae* pv. syringae strain 61, at the phenotypic and gene expression level (Yi et al., 2009).

Methods and Materials

Seeds of lima bean (*Phaseolus lunatus* L.) were collected from a natural population in the coastal area of the state of Oaxaca, Mexico (15°55′ N, 097°09′ W). *Pseudomonas syringae* pv. syringae strain 61 was kindly provided by Dr. Choong-Min Ryu, KRIBB, Daejeon, S.-Korea. The experiments were conducted in a greenhouse mimicking natural

light conditions and temperature (12/12 h, D/N at 30°C/26°C). Each three *Phaseolus lunatus* plants were placed in closed transparent boxes of 40 l volume and exposed over 6 or 24 h to one of the two volatiles, nonanal or MeSA (purchased from Sigma-Aldrich, México DF, Mexico). Stable concentrations of the volatile in the headspace were achieved by collocating into the chamber a Petri dish with 2 ml of a lanolin paste in which the compound had been dissolved. We used pastes at concentrations of 800 μg l^{-1} or 8 mg l^{-1} of nonanal and 4 mg l^{-1}, 8 mg l^{-1} or 80 mg l^{-1} MeSA. These concentrations were chosen based on earlier observations (Kost and Heil, 2006; Yi et al., 2009) to produce natural concentrations of the respective VOCs in the atmosphere. For comparison: 190 mg l^{-1} of MeSA in lanolin produced the concentration found in the headspace of a lima bean treated with jasmonic acid (Kost and Heil, 2006), whereas plants treated with benzothiadiazole to induce pathogen resistance emitted about three times more MeSA (Yi et al., 2009). After the exposure time, the Lanolin paste was removed and the boxes were ventilated. Plants were challenged (spray-inoculated) with the pathogen 5 d after VOC exposition to quantify resistance by extracting bacteria 4 d post challenge. Two leaves were collected from each plant, weighed, ground with mortar and pestle in 1 ml of sterilized distilled water, and the supernatant was diluted 1.10, 1:100, and 1:1000. The resulting suspensions were plated on Petri dishes containing KB agar medium with 100 μg ml^{-1} of rifampicin, to which the *P. syringae* strain 61 is resistant. The Petri dishes were incubated at 28°C for 48 h, to count colony forming units (CFUs). All experiments were conducted three times independently with three individual plants each.

Results and Discussion

Exposure to the headspace over a lanolin paste with 800 μg l^{-1} or 8 mg l^{-1} of nonanal for 6 h decreased infection rates significantly, and the same pattern emerged after an exposure over 24 h (Fig. 1a). By contrast, exposure to MeSA at a low concentration (4 mg l^{-1}) over 6 h did not significantly reduce bacterial infections, whereas the same concentration caused a significant effect after an exposure over 24 h (Fig. 1b). The dose–response relation that emerged after 6 h of exposure to MeSA (left part of Fig. 1b) disappeared in the 24 h treatment, in which the three tested concentrations of MeSA caused indistinguishable high levels of resistance to *P. syringae* (right part of Fig. 1b). A defined concentration of a potentially resistance-enhancing volatile caused a significant resistance effect only when the time of exposure was long enough. Three concentrations of MeSA, which had quantitatively different effects after 6 h, induced the same (high) level of resistance after

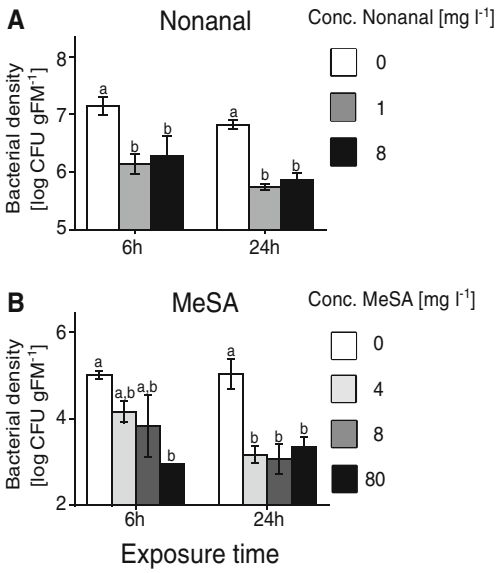

Fig. 1 Bacterial infection in lima bean plants exposed to resistance-inducing volatiles. Bacterial titres [in log (colony forming units) per gram leaf fresh mass] are displayed for *Phaseolus lunatus* plants that had been exposed over 6 or 24 h to different concentrations of nonanal (panel **a**) and methyl salicylate (MeSA: panel **b**). Sample size $N=3$ plants per concentration and exposure time; the entire experiment was repeated three times, with similar results. Bars display means ± standard errors, different letters above bars mark significant differences among treatments ($P<0.05$ according to least significant difference *post hoc* tests)

24 h of exposure. By contrast, the two concentrations of nonanal tested caused similar levels of resistance, independently of exposure time. In short, it depended on both the concentration and the chemical nature of the compound tested, whether the time of exposure affected the level of the resulting resistance.

Inhibitory effects of exogenous VOCs on the subsequent infection of plants are consistent with two different mechanisms: direct anti-microbial effects and the elicitation of plant-internal pathways for signal perception and transduction. Volatiles can be adsorbed on or in plant leaves (Himanen et al., 2010). Nonanal at a concentration of ca. 8 μg L^{-1} in the growing medium had inhibitory effects on the bacteria, *Bacillus cereus* and *Listaria monocytogenes* (Bisignano et al., 2001), and nonanal at 0.7 μg l^{-1} or MeSA at 0.6 μg l^{-1} in the gas phase inhibited the germination of conidia of the plant pathogenic fungus, *Colletotrichum lindemuthianum*, both *in vitro* and in bean plants (E. Quintana-Rodríguez and M. Heil, unpubl. data). However, Himanen et al. (2010) detected adsorbed hetero-specific VOCs within the first hours after exposing birch (*Betula pendula*) to other plants, whereas our plants were challenged 5 d after VOC exposition. It appears to be unlikely that exogenous VOCs remained in or on leaves over 5 days without being metabolized. By contrast, both VOCs primed resistance-related marker genes in *P. lunatus* under comparable experimental conditions (Yi et al., 2009)

and can thus be involved in a resistance induction. For MeSA, a conversion via salicylic acid-binding protein 2 (SABP2) into salicylic acid (SA) has been brought forward for tobacco (Park et al., 2007) and can be suspected also for lima bean. In this scenario, MeSA or the active hormone, SA, might accumulate in the leaf until a critical threshold level has been reached. For nonanal, the mechanism of its conversion into an active plant-internal signal remains a matter of speculation. We hypothesize that plant-plant signaling can depend on mechanisms that involve the accumulation of volatiles, or of a down-stream signal, in the receiving plant

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