



Serendipitous, cross familial discovery of the first long-range chemical attractants for antlions (Neuroptera: Myrmeleontidae): (1*R*,2*S*,5*R*,8*R*)-iridodial and *Z,E*-nepetalactol

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Synthetic (1*R*,2*S*,5*R*,8*R*)-iridodial, the key pheromone component of many green lacewings in the genus *Chrysopa*, strongly attracted adult males and females of the North American antlion, *Dendroleon speciosus* Banks. In addition, one of the common sex pheromone components of many aphids to which *Chrysopa* spp. are weakly attracted, *Z,E*-nepetalactol, was also weakly attractive to *D. speciosus* adults. Iridodial and *Z,E*-nepetalactol also elicited strong and weak electroantennogram detector (EAD) responses, respectively, in *D. speciosus* adults. Previously reported semiochemicals from European and Israeli antlion species did not elicit EAD or behavioral responses from *D. speciosus* adults. The earlier studied antlions release volatile chemicals from male-specific metathoracic glands associated with structures on the hind wings (Eltringham's organs) that are thought to enhance evaporation of the secretion. Although *D. speciosus* males have Eltringham's organs similar to those of other antlion species, we discovered that *D. speciosus* males have a pair of white tubular glands that extend posteriorly into the abdomen, opening in the resting pits of the Eltringham's organs. Further gas chromatograph (GC)-EAD analysis of another commercially available antlion species, *Myrmeleon crudelis* Walker, showed that this species did not respond to the lacewing or aphid related volatile compounds, but strongly responded to the reported antlion semiochemicals, namely, nerol, 10-homonerol, and nerol oxide. The male-specific abdominal glands of *D. speciosus* are presumably pheromone glands, but this hypothesis requires verification in the future.

Keywords: antlion, lacewing, pheromone, attractant, iridodial, nepetalactol, trap, GC-EAD

INTRODUCTION

With more than 1500 described species worldwide (Strange, 2004), the Myrmeleontidae (called antlions) is the largest family in the order Neuroptera. Antlions exploit a range of habitats; their larvae are sedentary predators living in tree holes, on the soil surface, and in specialized pits they create in sandy soil (Mansell, 1999). In contrast to the rich knowledge of larval behaviors (Lambert et al., 2011; Scharf et al., 2011), due to the short life span and nocturnal activity of adults their behavior is less well known (Yasseri and Parzefall, 1996; Güsten, 2002; Szentkirályi and Kazinczy, 2002; Penny et al., 2007).

In particular, semiochemicals mediating the behavior of myrmeleontids are poorly known. Indeed, semiochemicals have been identified from only three sympatric European antlion species [*Eurleon nostras* (Fourc.), *Grocus bore* Tjed. [now *Myrmeleon bore* (Tjed.)], and *Myrmeleon formicarius* L.], and two sympatric species in Israel (*Synclisis baetica* Rambur, and

Acanthacilicis occitanica Villers) (Bergström, 2008). These antlion species belong to four different genera. In the antlion species studied thus far, volatile chemicals are produced in paired male-specific thoracic glands, from which a total of six structurally similar compounds have been identified: two monoterpene alcohols (nerol and 10-homonerol); their corresponding oxides (nerol oxide and 10-homonerol oxide), and two mono-unsaturated secondary alcohols [(*R*)-(*Z*)-6-tridecen-2-ol and (*R*)-(*Z*)-6-undecen-2-ol]. Males of each species produce specific two-component blends thought to function in reproductive isolation (Elofsson and Löfqvist, 1974; Löfqvist and Bergström, 1980; Bäckström et al., 1989; Bergström et al., 1992). The roles of male-specific volatiles for the three European species were further tested by gas chromatography-electroantennogram detector (GC-EAD) and Y-tube bioassay experiments, which suggested that these secretions are sex or aggregation pheromones (Yasseri et al., 1996, 1997, 1998; Yasseri and Parzefall, 1996; Bergström,

2008). However, no long-range sex or aggregation attractants for any antlions have been positively elucidated.

In the course of testing synthetic pheromone candidates for the green lacewing, *Chrysopa nigricornis* Burmeister (Zhang et al., 2006a), an interesting discovery was made, namely, that both males and females of the antlion, *Dendroleon speciosus* Banks (Myrmeleontidae: Dendroleontinae) were significantly attracted to several of the lacewing treatments tested during late July and mid-August 2004. *D. speciosus* was previously unknown in the state of Washington (Penny, personal communication).

In the current paper, we report (1) the identity of two long-range attractants for *D. speciosus*, (2) electrophysiological responses of *D. speciosus* adults to reported lacewing and antlion semiochemicals, (3) behavioral responses of *D. speciosus* adults to reported lacewing and antlion semiochemicals in the field, and (4) the discovery of a unique orientation of male-specific glands in *D. speciosus*. In addition, the electrophysiological responses to previously known lacewing and antlion semiochemicals were tested on a common commercially available antlion species, *Myrmeleon crudelis* Walker.

MATERIALS AND METHODS

INSECTS AND CHEMICALS

Live adult *D. speciosus* males and females for GC-EAD studies, and dead specimens for gland dissections, were collected from sticky traps baited with green lacewing pheromone (Zhang et al., 2004), whereas *M. crudelis* adults for GC-EAD recordings were reared from commercially available larvae (<http://www.antlionfarms.com/>).

Chemical standards were obtained commercially or synthesized. (1*R*,2*S*,5*R*,8*R*)-Iridodial [80%; with 20% of (1*R*,2*S*,5*R*,8*S*)-iridodial as an impurity] was synthesized as previously described (Chauhan et al., 2004). *Z,E*-Nepetalactone (98%) and *E,Z*-nepetalactone (96%) were isolated from commercially available catnip oil via a pH-sensitive chemical separation technique (Chauhan and Zhang, 2008). *Z,E*-Nepetalactol (~90%) was prepared by NaBH₄ reduction of *Z,E*-nepetalactone as previously described (Chauhan et al., 2004). Nerol (>97%) and methyl salicylate (99%) were from Sigma-Aldrich, St. Louis, MO, USA, and nerol oxide (>95%) was purchased from Bedoukian Research Inc., Danbury, CT, USA. 10-Homonerol (98%) and 10-homonerol oxide (97%) were synthesized as described by Baeckstrom et al. (1982) and Cahiez et al. (1976), respectively. (*Z*)-6-Tridecen-2-ol (90%) and (*Z*)-6-undecen-2-ol (90%) were synthesized according to Clososki et al. (2004). The Benallure™ dispensers were purchased from Gardens Alive, Inc., Lawrenceburg, IN, USA.

GC-EAD ANALYSIS

Electrophysiological responses by antennae of three *D. speciosus* (both sexes) and three *M. crudelis* (females only) to a synthetic mixture including several green lacewing and antlion semiochemicals (100 ng/μl each in hexane) were recorded in splitless mode using a Varian CP-3800 GC equipped with a polar column (HP-INNOWAX; 30 m × 0.53 mm × 1.0 μm; Agilent Technologies, Wilmington, DE, USA), using a 1:1 effluent splitter that allowed simultaneous flame ionization detection (FID) and EAD of the

separated volatile compounds. Helium was used as the carrier gas, and the injector temperature was 220°C. Column temperature was programmed from 50°C for 2 min, rising to 240°C at 10°C/min, then held for 10 min. The outlet for the EAD was held in a humidified 0.5-m/s air stream over an antlion antennal preparation. EAD recordings were made using silver wire-glass capillary electrodes filled with Beadle–Ephrussi Ringer solution (Zhang et al., 2000) on freshly cut antennae. Antennal signals were stored and analyzed on a PC equipped with a serial IDAC interface box, using an EAD ver. 2.5 Program (Ockenfels SYNTECH GmbH, Kirchzarten, Germany).

DISSECTIONS OF EXOCRINE GLANDS

D. speciosus adults (both sexes) were carefully removed from the sticky traps, and kept in 70% ethanol. Dissections of these antlion specimens in search of exocrine glands were conducted under a stereomicroscope (Fisher Scientific), in a small glass Petri dish filled with tap water. Photos of these dissections were taken using an iPhone 5 camera through one of the microscope oculars.

FIELD TRAPPING

Three field-trapping experiments were carried out from late July through mid-September 2004, 2006, and 2010, either in a home garden or small orchards in and around Spokane, WA, USA. Pherocon VI traps with removable sticky inserts (Trécé Inc., Adair, OK, USA) were hung 1.5–2.0 m above ground on either garden stakes, fence posts or the branches of cherry or apple trees, ca. 5–10 m apart within each trap line. For each experiment, two or three sets of traps (each set contained all the tested treatments) were deployed, and the initial trap positions were randomized within a set. Traps were visited once a week, at which time the sticky inserts were replaced, and trap positions were re-randomized. The sticky inserts with captured insects were taken to the laboratory for recording of the species and gender of trapped specimens.

Experiment 1 (with two sets of sticky traps), conducted at Strawberry Hill Farm from 28 July to 24 August 2004, compared the lacewing pheromone [(1*R*,2*S*,5*R*,8*R*)-iridodial] with *Z,E*-nepetalactone, *Z,E*-nepetalactol, and the combination of iridodial with methyl salicylate; 5 mg of each compound in 50 μl hexane was loaded onto rubber septa, each of which were inserted into a 2-ml open plastic centrifuge-tube. A commercial beneficial insect attractant, Benallure™ (Gardens Alive Inc.), was also included in each trap line for comparison. In Experiment 2, three sets of sticky traps were deployed at three locations (Prestini, Arbor Crest, and Morgan) from 1 to 29 August 2006, to test different dosages of iridodial (0, 0.1, 1, 2, 5, 10 mg, each in 100 μl hexane) applied to 2.5 × 4.5 × 0.4 cm pieces of felt each sealed in a 0.15 mm polyethylene (PE) bag (3 × 5 cm). In Experiment 3, three sets of sticky traps were deployed at two locations (two sets at Mt. St. Michaels, and one set at Morgan) from 28 July through mid-September 2010, to test the responses of the native antlion species, *D. speciosus*. Lures tested in Experiment 3 included reported potential antlion pheromone candidates in binary blends (neat compounds loaded onto rubber septa inserted into a 2-ml open plastic centrifuge-tube), and the green lacewing pheromone, iridodial (2 mg in 100 μl hexane

loaded onto rubber septa; inserted into a 2-ml open plastic centrifuge-tube).

STATISTICAL ANALYSIS

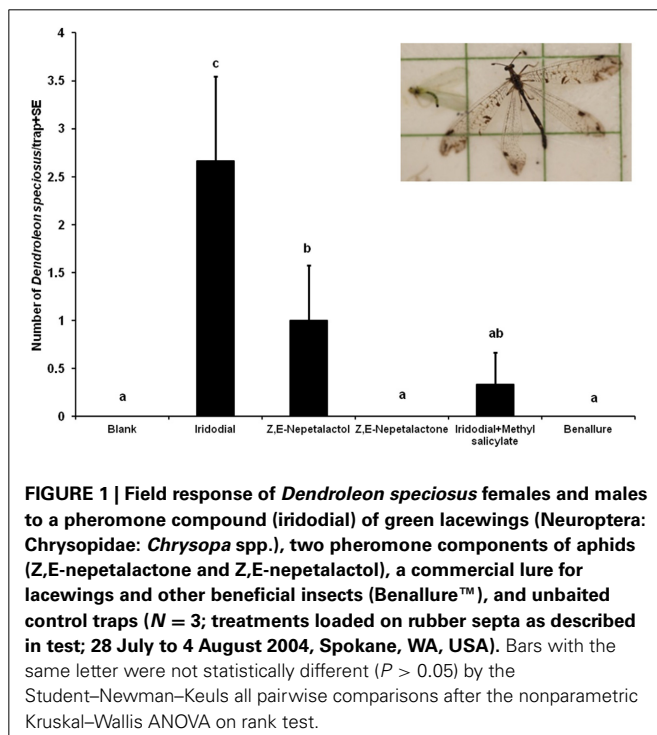
Because of heterogeneity of variances among treatments, trap catch data (number of antlions or lacewings/trap) were analyzed using the nonparametric Kruskal–Wallis ANOVA on rank test, followed by the Student–Newman–Keuls all pairwise comparison to separate means (Zar, 1984).

RESULTS

FIELD TRAPPING

The results of Experiment 1 on green lacewings were reported in Zhang et al. (2006a). In this experiment, iridodial resulted in significantly higher trap catches of males for both green lacewing species (*C. nigricornis* and *C. oculata*) than did *Z,E*-nepetalactol, whereas addition of methyl salicylate to iridodial significantly increased trap catches for *C. nigricornis* but not for *C. oculata* (see Figure 4 in Zhang et al., 2006a). Surprisingly, significant numbers of male and female *D. speciosus* adults were also captured in traps baited with iridodial and *Z,E*-nepetalactol during late July and mid-August 2004, with iridodial being significantly more attractive than *Z,E*-nepetalactol (Figure 1). Addition of methyl salicylate to iridodial significantly decreased the antlion trap catches to levels not different from the unbaited blank control traps (Figure 1). *Z,E*-nepetalactone and the commercial beneficial attractant, Benallure™, were both inactive.

In Experiment 2, *D. speciosus* adults were significantly attracted to sticky traps baited with PE-bag dispensers containing ≥ 1 -mg of iridodial. The 2-mg dose was the most attractive, followed by the 5-mg loading; further increase in dosage (e.g.,



10-mg) resulted in significant decreases in trap catch (Figure 2). The lowest dosage loaded, 0.1-mg, was inactive for *D. speciosus*. In the same experiment, significant numbers of the golden-eyed lacewing (*C. oculata*) males were also captured in traps baited with ≥ 1 -mg of iridodial, with no differences in trap catches among the 1 to 10-mg dosages (Figure 2).

In Experiment 3, none of the reported potential antlion pheromone binary blends were attractive to *D. speciosus* or to green lacewings (Table 1). However, traps baited with the lacewing pheromone, iridodial (2-mg loading), captured significant numbers of antlions (both sexes) and green lacewings (males) (Table 1). The overall sex-ratio of captured antlions was 1:1.3 ($\text{♀} : \text{♂}$), whereas only males of the green lacewings were captured.

GC-EAD

Antennae of *D. speciosus* (from both males and females) strongly responded to the green lacewing pheromone component, iridodial, and weakly (but consistently) to *Z,E*-nepetalactol (Figure 3; upper and middle EAD traces). No antennal responses were elicited from males or females of *D. speciosus* to nepetalactones, or to any of the three reported antlion candidate pheromone components, nerol, 10-homonerol, and nerol oxide. In contrast to *D. speciosus*, the commercially available *M. crudelis* females showed no antennal activities to any of the green lacewing attractants, but demonstrated strong EAD responses to all three reported antlion pheromone candidates (Figure 3, lower EAD trace).

DISSECTIONS OF EXOCRINE GLANDS

Adult *D. speciosus* males possess yellowish-colored, paired Eltringham's organs (EO) (Eltringham, 1926) (also called axillary pilaris; Yasseri et al., 1998) at the base of their hindwings (EO; Figure 4A). Dissection of males revealed a pair of 8–10 mm

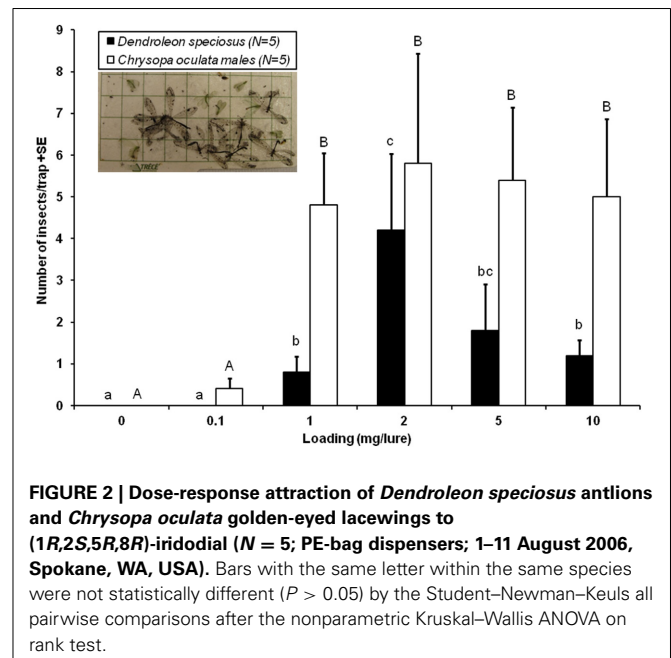
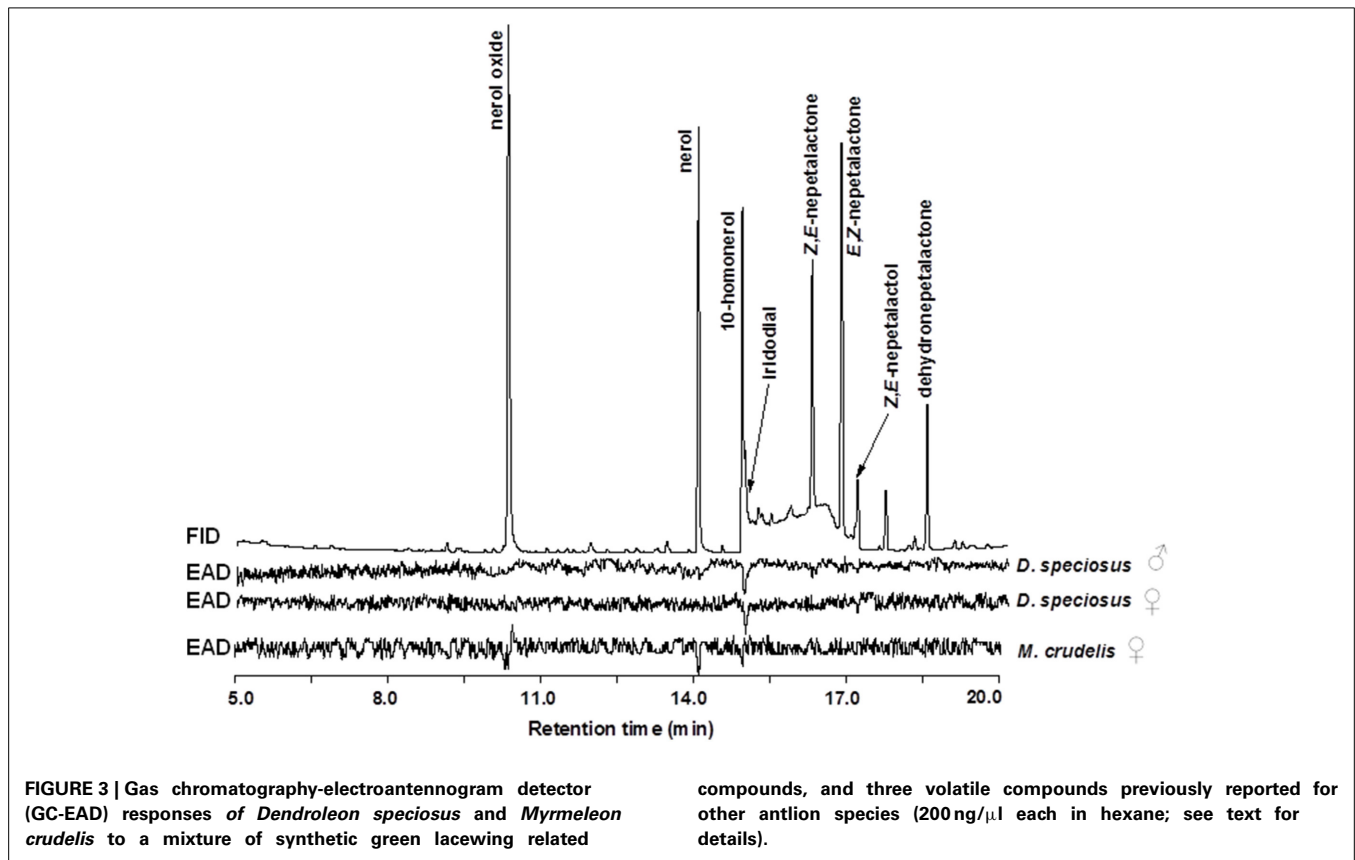


Table 1 | Mean trap catches (#/trap/collection; $N = 9$) of the antlion, *Dendroleon speciosus*, and the golden-eyed lacewing, *Chrysopa oculata*, to previously reported antlion semiochemical candidates and the green lacewing pheromone, iridodial; July 28th to September 16th, 2010, Spokane, WA, USA.

Treatment	Antlion attractant candidates (mg/rubber septum)						# of <i>D. speciosus</i>	# of <i>C. oculata</i>	
	Nerol oxide	Nerol oxide	10-Homonerol oxide	10-Homonerol oxide	(Z)-6-Undecen -2-ol	(Z)-6-Tridecen -2-ol			Iridodial
A	10		10					0 ± 0 ^a	0.0 ± 0 ^a
B		10			10			0 ± 0 ^a	0.0 ± 0 ^a
C		10				10		0 ± 0 ^a	0.2 ± 0.2 ^a
D		10		10				0 ± 0 ^a	0.0 ± 0 ^a
E				10	10			0 ± 0 ^a	0.2 ± 0.2 ^a
F	10	10						0 ± 0 ^a	0.0 ± 0 ^a
G							2	1.33 ± 0.4 ^b	7.3 ± 1.4 ^b
H(blank)								0 ± 0 ^a	0.0 ± 0 ^a

Means with the same letter within the same species were not statistically different ($P > 0.05$) by the Student–Newman–Keuls all pairwise comparisons after the nonparametric Kruskal–Wallis ANOVA on rank test.



long white tubular glands extending from the resting pits for Eltringham's organs in the first abdominal segment posteriorly nearly 1/2 length of the abdomen (Figure 4C). Eltringham's organs and the abdominal glands (AGs) are absent in *D. speciosus* females (Figures 4B,D).

DISCUSSIONS

This is the first demonstration of long-range attractants for any antlion species. Our results clearly show that the male-produced green lacewing pheromone, (1R,2S,5R,8R)-iridodial (Chauhan et al., 2004, 2007; Zhang et al., 2004, 2006a,b), is also

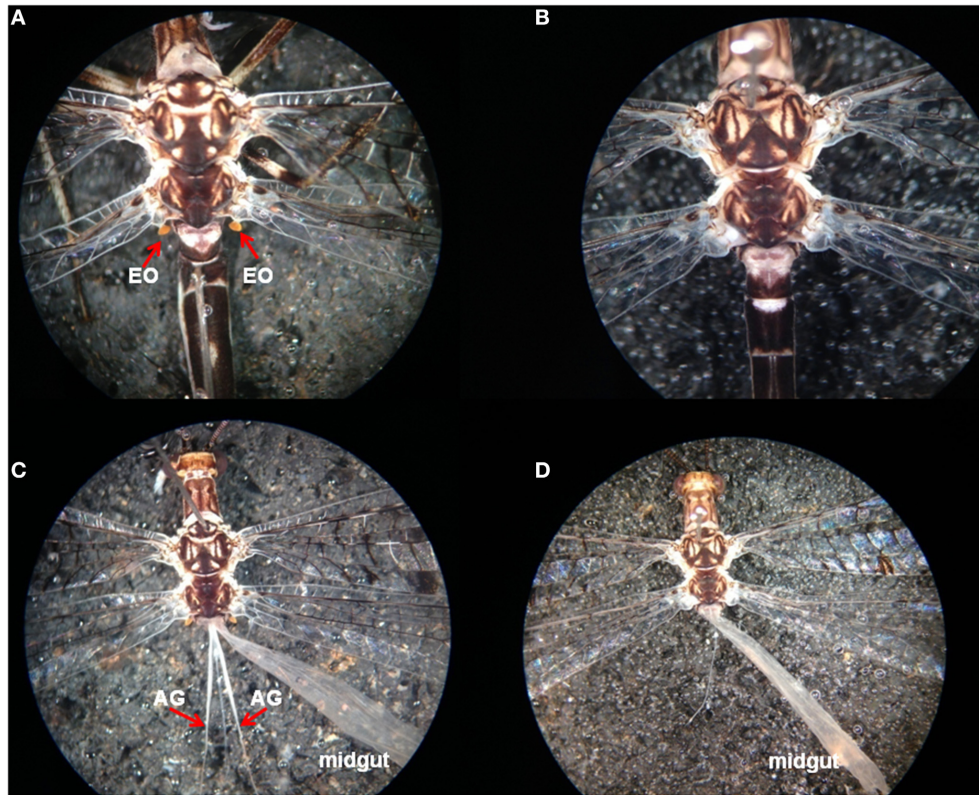


FIGURE 4 | (A) Male-specific Eltringham's organs (EOs; red arrows) of *Dendroleon speciosus*; **(B)** Shows that the EOs are missing from *D. speciosus* females; **(C)** Shows the male-specific abdominal glands (AGs;

red arrows) opening at the base of the resting pits for the EOs, extending posteriorly along the midgut; **(D)** Show that the AGs are missing from *D. speciosus* females.

a strong attractant for both sexes of the antlion, *D. speciosus*. *Z,E*-Nepetalactol, which is a known sex pheromone component for many aphids (Pickett et al., 2013; Han et al., 2014), is also a weak attractant for *D. speciosus* adults. Iridodial and *Z,E*-nepetalactol also elicited a strong and a weak antennal electrical response, respectively, from *D. speciosus* adults indicating the existence of olfactory receptor neurons on the antlion antennae for these two volatile compounds. This finding revealed an interesting cross-attraction between species in different families within the same order. No fresh live *D. speciosus* adults were available for volatile collections and chemical analyses; the only live *D. speciosus* adults, which were not suitable for pheromone collection, were used for GC-EAD study after being taken from the sticky traps baited with the lacewing pheromone. Whether or not *D. speciosus* use the same or similar volatile compounds as its pheromone component(s) or use the lacewing pheromone as a kairomone, remains unknown.

There are about 20 described species of *Dendroleon* in the world, with nine species in China, four species in Australia, three species in North America, and one species each in Europe, Madagascar, Japan, and Java (Strange, 2004; Stange, 2008; Wang and Wang, 2008; Zhan et al., 2012). Only two *Dendroleon* species occur in the USA; *D. speciosus* in the west (west of 100° Meridian), and its allopatric counterpart, *D. obsoletus* (Say), in the eastern USA (east of 100° Meridian) (Stange, 2008). Larvae of *D. speciosus*

have been found in northern California living in tree holes of pine and oak at low elevations (<600 m), and on rain and snow protected rock shelves at higher elevations (Stange, 2008). The larvae have also been found in houses near suitable forest habitats. According to Miller (1990) (cited in Stange, 2008, but not listed in the reference list) adult females of *D. speciosus* appear to be unique in this family in that they die shortly after laying their eggs instead of continuing to feed and developing more eggs. Based on our trapping results in three different years, adults of *D. speciosus* fly mainly during late July to mid-August in the Spokane area of Washington, which is in agreement with collection dates from western states in USA (listed in Stange, 2008), and new distributional records in British Columbia, Canada (Meinander et al., 2009).

As mentioned in the Introduction herein, antlions studied in Europe and Israel produce volatiles in a pair of male-specific thoracic glands (Elofsson and Löfqvist, 1974; Löfqvist and Bergström, 1980; Bergström et al., 1992; Bergström, 2008). These glands open ventrally at the junction of the thorax and abdomen. In *E. nostras* and *M. bore*, but not from *M. formicarius*, males have club-like projections (brush-like tufts; called Eltringham's organs) (Eltringham, 1926) from the posterior margin of the hind-wings. Eltringham's organs have been considered to be the dispersing organs for substances from the thoracic glands (Elofsson and Löfqvist, 1974; Löfqvist and Bergström, 1980).

However, a morphological study by Güsten (1998) indicated that antlion thoracic glands are much more variable than thought from the few species of Myrmeleontini and Acanthaclisini initially investigated; species have now been found in the tribes Myrmecaelurini and Nemoleontini in which the thoracic glands are equally developed in both sexes.

The male-specific thoracic gland volatiles identified from three European species were also tested electrophysiologically and behaviorally in the laboratory (Yasseri et al., 1996, 1997, 1998). In female choice tests using a Y-tube olfactometer, solitary males were not able to attract females; however, females were strongly attracted by groups of males, indicating that male-produced volatiles might act as aggregation pheromones as part of lek formation. In the initial experiment, an occasional GC-EAD response by antenna of one *M. bore* female to conspecific male-specific thoracic gland volatiles [10-homonerol and (*Z*)-6-undecen-2-ol] was observed, but was not reproducible; likely this was due to the short life of antlion antennal contact gel electrode preparations (Yasseri et al., 1998). A previously undiscovered additional gland, opening between the meta- and mesothorax, was found in three antlion species (both sexes): *E. nostras*, *M. bore*, and *M. formicarius*. Analysis of the secretions of *M. bore* and *E. nostras* showed a lower concentration and different chemical compositions (dodecyl acetate and acetogenine in *M. bore* females; and hexadecane acid in *E. nostras* females) compared to compounds identified from the male-specific metathoracic glands (Yasseri et al., 1998). It is not known if these new chemical compounds are behaviorally active alone or with the two component blends identified earlier (Löfqvist and Bergström, 1980; Bergström et al., 1992). Thus far, there is no behavioral data on the function of male-produced binary blends in the two Israeli antlion species (*S. baetica* and *A. occitanica*) that have been investigated (Bergström, 2008).

In *D. speciosus* males, we not only observed the yellowish, paired Eltringham's organs at the base of the hindwings (EO; Figure 4A), but we also found a pair of long white tubular glands (Figure 4C) opening in the resting pits of the Eltringham's organs. Unlike the thoracic glands reported earlier for the European and Israeli antlion species (extending anteriorly into the thorax), the paired tubular glands in *D. speciosus* males, which we are designating abdominal glands (AGs), extend posteriorly into the abdomen (Figure 4C). Both the Eltringham's organs and the AGs are absent in *D. speciosus* females (Figures 4B,D). It is likely that these highly developed male-specific glands in *D. speciosus* males are the site of pheromone production; confirmation of this supposition is needed. Furthermore, the Eltringham's organs might function as the dispersers for substances released from the AGs. A similar male-specific AG system (with four narrow tubular glands extending posteriorly into the abdomen) was found earlier in *Brachynemurus longicaudus* (Burmeister) of the tribe Brachynemurini (Güsten, 1998). The green lacewing (*Chrysopa* spp.) pheromone, (1*R*,2*S*,5*R*,8*R*)-iridodial, is produced from male-specific abdominal glands, but these pheromone glands are elliptical epidermal glands that are abundantly distributed on the third–eighth abdominal sternites (Zhang et al., 2004). *Chrysopa* and other genera of green lacewings also possess thoracic glands in both sexes that produce compounds, some of

which are obnoxious to the human nose, presumably for defense (Aldrich et al., 2009). No long-range pheromones or attractants have yet been reported for other Neuropteran families.

In summary, our GC-EAD experiments showed that *D. speciosus* antlions do not have olfactory receptors on their antennae for nerol, 10-homonerol and nerol oxide, three antlion pheromone candidates known from some European and Israeli antlion species representing four genera from two subfamilies (Myrmeleontinae and Acanthaclisinae). On the other hand, antennae of female adults of the Nearctic *M. crudelis* (Myrmeleontinae) responded strongly to the three reported antlion pheromone candidates (nerol, 10-homonerol and nerol oxide), indicating that this species might use the same or similar volatile compounds for intra- and/or inter-specific chemical communication. The fact that *D. speciosus* larvae do not make sand pits to trap ants, as do the previously studied antlion species, and that adults of this species exhibit a strong interfamilial attraction to a key pheromone component of certain green lacewings rather than the known congeneric semiochemicals, suggests that dendroleontine antlions are not closely aligned with antlions whose chemical communication was studied earlier.

AUTHOR CONTRIBUTIONS

Designed research: Qing-He Zhang and Rodney G. Schneidmiller. Performed research: Qing-He Zhang, Guiji Zhou, Doreen R. Hoover, Neil J. Michaelson, Paul Bryant, Armenak Margaryan, Kamlesh Chauhan, Jeffrey R. Aldrich, and Rodney G. Schneidmiller. Analyzed data: Qing-He Zhang, Guiji Zhou, Doreen R. Hoover, Neil J. Michaelson, Paul Bryant, Armenak Margaryan, Kamlesh Chauhan, Jeffrey R. Aldrich, and Rodney G. Schneidmiller. Statistical analyses: Qing-He Zhang and Doreen R. Hoover. Wrote the paper: Qing-He Zhang and Jeffrey R. Aldrich.

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