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Prothoracic Gland Semiochemicals of Green Lacewings

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Abstract Adult chrysopids have paired prothoracic glands (PG) that are thought to produce defensive secretions (allomones). We analyzed PG extracts of the following green lacewings from North and South America, Australia, and China: *Ceraeochrysa cubana* (Brazil); *Chrysopa* (= *Co.*) *oculata, Co. nigricornis, Co. incompleta, Co. quadripunctata* (USA), and *Co. septempunctata* (China); *Chrysoperla* (= *Cl.*) *rufilabris* (USA) and *Cl.* sp. (Brazil); *Plesiochrysa ramburi* and *Mallada* spp. (Australia). PG secretions are characteristic for species within a genus, except for *Chrysopa* spp. *(Z)*-4-Tridecene is ubiquitous, but (*Z,Z*)-4,7-tridecadiene is a major PG constituent in some *Chrysopa* spp. and in *P. ramburi*. Earlier reports that *Co. oculata* and *Co. nigricornis* produce 1-tridecene were shown to be in error. *Chrysopa* PG secretions are distinguished by the presence or absence of N-3-methylbutylacetamide, plus skatole (3-methylindole). Skatole is also identified for the first time from the *Plesiochrysa* and *Ceraeochrysa*. The PG secretion in *Plesiochrysa ramburi* is characterized by the presence of *(Z)*-4-undecene instead of *(Z)*-4-tridecene, and N-3-methylbutylpropanamide instead of the acetamide, resembling the PG secretions of *Chrysopa nigricornis, Co. septempunctata* and *Co. incompleta*. The chemotaxonomic value of PG semiochemicals is discussed, including evidence for subgroups within the genus *Chrysopa* as it now stands.

Keywords Pheromone · Allomone · Tridecene · Skatole · Iridodial · Amide · Chemotaxonomy · Predator · Biosynthesis · Neuroptera · Chrysopidae

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Introduction

Green lacewing larvae are voracious predators of small soft-bodied arthropods, such as aphids and mites, and are sold commercially, principally for aphid control in greenhouses (Stelzl and Devetak 1999; McEwen et al. 2001; Henry and Wells 2007). In some chrysopid genera, the adults are also predacious (e.g., *Chrysopa*), but many adult green lacewings

(e.g., *Chrysoperla*) subsist on only nectar and pollen (Brooks and Barnard 1990).

Green lacewing adults have long been known to communicate intraspecifically via substrate vibrations (e.g., Henry 1982), but only recently was it discovered that males in the genus *Chrysopa* produce aggregation pheromones from thousands of elliptically shaped glands embedded in their abdominal cuticle (Chauhan et al. 2004; Zhang et al. 2004, 2006a, b). Interestingly, female goldeneyed lacewings [Neuroptera: Chrysopidae: *Chrysopa* (= *Co.*) *oculata* Say] do not enter traps baited with pheromone (1*R*,2*S*,5*R*,8*R*-iridodial) (Chauhan et al. 2004; Zhang et al. 2004), presumably because females attract males via substrate-borne vibrations at close range (Henry 1982). However, Chauhan et al. (2007) showed that significant numbers of wild *Co. oculata* females are attracted to the vicinity of iridodial dispensers in the field, and observed that the attracted females laid eggs on soybean leaves near the dispensers. Thus, the judicious application of *Chrysopa* pheromones may provide a practical means to conserve and augment these lacewings in the field for biological control of pests.

All adult chrysopids possess paired prothoracic glands (PG) thought to produce defensive secretions (allomones) (Güsten and Dettner 1991; Szentkirályi 2001). In the course of ongoing research efforts to find aggregation pheromones for other chrysopid species, we have investigated or reinvestigated the PG secretions of ten species from five genera in the tribe Chrysopini. Blum et al. (1973) identified skatole (3-methylindole) as the compound responsible for the stench of adult goldeneyed lacewings (*Co. oculata*), with the major non-odorous (to humans) component of the secretion (93%) reportedly being 1-tridecene. The presence of skatole in PG secretions of *Chrysopa* spp. was verified by Güsten and Dettner (1991), but these authors found *(Z)*-4-tridecene in species representing five genera of Chrysopini, including six *Chrysopa* species. However, Güsten and Dettner (1991) did not include spectral data or chemical details as to how the double bond position and geometry of tridecene were determined. More recently, Zhu et al. (2000) identified *(Z)*-4-tridecene as the sole major constituent of the PG secretion in *Cl. carnea*, but they also described the secretion as "offensive-smelling", which is enigmatic since skatole was not present and tridecenes are not strong or foul smelling.

Here, we report on analyses of PG secretions for the following green lacewings from North and South America, Australia, and China: *Ceraeochrysa* (= *Ce.*) *cubana* (Hagen) (Brazil); *Chrysopa* (= *Co.*) *oculata* Say, *Co. nigricornis* Burmeister, *Co. incompleta* Banks, *Co. quadripunctata* Burmeister (USA), and *Co. septempunctata* Wesmael (China); *Chrysoperla* (= *Cl.*) *rufilabris* (Burmeister) (USA) and *Cl.* sp. (Brazil); *Plesiochrysa ramburi* (Schneider) and *Mallada* spp. (Australia).

Methods and Materials

Insects and Preparation of Extracts The sources, method of sampling the adult insects, identity, and the specialists responsible for identifying the lacewings are listed in Table 1. Identifications were aided by reference to Penny et al. (2000) and Brooks and Barnard (1990). Traps used to catch live chrysopids were modeled after a previously described trap (Aldrich et al. 1984). Iridodial lures were prepared as described before (Chauhan et al. 2007), except that octane was used as solvent instead of heptane. The plant volatile lure was prepared in an analogous manner using β-caryophyllene/ 2-phenylethanol/ methyl salicylate (1:1:1 by volume; total active ingredient=5 mg). Chemical extracts were prepared in ca. 50 µl of methylene chloride (EMD Chemicals Inc., Gibbstown, NJ, USA) by either extracting the whole thorax (earlier samples) or by excising the glands from the prothorax (later samples). The paired prothoracic glands were easily removed by fastening a lacewing, dorsal side up, under water in a dissecting dish, transversely cutting the dorsal intersegmental membrane between the prothorax and mesothorax, and making a dorso-medial incision in the prothorax exposing the bluish-colored glands in each side of the prothorax. Each gland, attached to a small piece of cuticle, was then removed with fine forceps, gently dried with tissue paper, and extracted in about 20 µl of solvent (CH_2Cl_2 or hexane) for analysis.

Chemical Standards The following commercially available standards were used: 1-tridecene, hexanoic acid, octanoic acid, skatole, 3-hexanol, 2-hexanol, tridecanol, benzaldehye, 2-phenylethanol, methyl salicylate (Aldrich Chemical Co., Milwaukee, WI, USA); nonanoic acid (Emery Industries, Cincinnati, OH, USA); nonanal, decanal, and β-caryophyllene (Bedoukian Research, Danbury, CT, USA). N-3-Methylbutylacetamide and N-3-methylbutylpropanamide were prepared by reactions of 3-methylbutylamine with acetyl and propanyl chlorides, respectively, at 0°C in the presence of triethylamine (Heath and Landolt 1988). *(Z,Z)*-4,7-Tridecadiene was synthesized starting with a −78°C suspension of hexyltriphenylphosphonium bromide (1.04 g, 2.44 mmol) in 20 ml of THF to which a 1M THF solution of lithium bis (trimethylsilyl)amide (2.3 ml, 2.3 mmol) was added. The reaction mixture was warmed to −30°C over 30 min, kept at this temperature for 1 h, and then cooled to −78°C. To this clear orange ylide solution, a 0.5 ml THF solution of *(Z)*-3-hepten-1-al [112 mg, 1 mmol; prepared by Swern oxidation (Chauhan et al. 1994) of *(Z)*-3-heptene-1-ol] was added. The reaction mixture was warmed to −30°C over 1 h, maintained at this temperature for 3 h, then quenched with 5 ml of a 25% ammonium acetate solution. The mixture was extracted with hexane (3×15 ml), and the combined extracts were washed with water (2×10 ml) and brine (10 ml), and then dried. All

Table 1 Chrysopids used for analyses of prothoracic gland secretions

Species[a]	Sex	No.	Date[b]	Location	Collection/Sampling Method
Chrysopa oculata	M	5	5 July	Beltsville, MD	Iridodial trap/Gland dissection
	M	4	27 Sept.	Beltsville, MD	Iridodial trap/Gland dissection
Chrysopa nigricornis	M	2	12 June	Spokane, WA	Iridodial lure/Gland dissection
	M	10	7 August	Spokane, WA	Fluorescent light[c]/Gland dissection
Chrysopa incompleta	M	8	24 Sept.	Byron, GA	Iridodial trap/Gland dissection
Chrysopa quadripunctata	M	2	31 May	Hyattsville, MD	Mercury vapor light/Thoracic extract
Chrysopa septempunctata	M	30	20 Aug.	Shenyang, China	Iridodial trap/ Thoracic extract
Ceraeochrysa cubana	M	5	19 April	Recife, Brazil	Laboratory colony/Thoracic extract
	F	5	19 April	Recife, Brazil	Laboratory colony/Thoracic extract
Chrysoperla rufilabris	M	5	8 Aug.	Hyattsville, MD	Mercury vapor light/Gland dissection
	F	4	8 Aug.	Hyattsville, MD	Mercury vapor light/Gland dissection
	M	3	22 Sept.	Byron, GA	Plant volatile trap/Gland dissection
***Chrysoperla* sp.**	M	5	17 April	Brasília, Brazil	Sweep netting/Thoracic extract
	F	5	17 April	Brasília, Brazil	Sweep netting/Thoracic extract
Plesiochrysa ramburi	F	1	8 Nov.	Brisbane, Australia	Sweep netting/Gland dissection
Mallada sp.	F	3	17 Nov.	Brisbane, Australia	Sweep netting/Gland dissection

[a] Determinations: *Co. oculata*, Dr. Oliver S. Flint, Jr., Section of Entomology, Smithsonian Institution, Washington D.C.; *Co. nigricornis*, Dr. N. D. Penny, Department of Entomology, California Academy of Sciences, San Francisco, USA; *Co. quadripunctata*, Dr. Catherine A. Tauber, Department of Entomology, Comstock Hall, Cornell University, Ithaca, NY, USA; *Co. septempunctata*, Dr. Baoyu Han, Tea Research Institute of Chinese Academy of Agricultural Sciences, Hangzhou, China; *Co. incompleta* and *Cl. rufilabris*, Dr. Ted E. Cottrell, USDA-ARS Southeastern Fruit & Nut Research Laboratory, Byron, GA; *Ceraeochrysa* and *Cl.* spp., Dr. Sérgio de Freitas, Universidade Estadual Paulista, Jaboticabal, São Paulo, Brazil; *Plesiochrysa* and *Mallada* spp., Dr. Shaun L. Winterton, Entomology Collection, Queensland Department of Primary Industries and Fisheries, Brisbane, Australia.

[b] All collected in 2007, except *Co. septempunctata* was collected in 2005.

[c] 13 watt Neolite® NL-13127 (Litetronics International, Inc., Alsip, IL, USA).

volatiles were removed *in vacuo*. Preparative thin layer chromatography (SiO_2, ethyl acetate/hexane, 1:7) gave *(Z,Z)*-4,7-tridecadiene (138 mg, 76%) and *(Z,Z)*-4,7-tridecadiene (6 mg, 3%). (*Z*)-4-Undecene, (*Z*)-4-tridecene, and (*Z*)-6-tridecene were synthesized by following the Wittig reaction procedure described above for *(Z,E)*-4,7-tridecadiene. The following reactions and products were obtained: the ylide of *n*-butyltriphenyl phosphonium bromide with heptanal produced 92% 4-undecenes (*Z*:*E,* 98:2); the ylide of *n*-butyltriphenyl phosphonium bromide with nonanal yielded 98% 4-tridecenes (*Z*:*E,* 93:7); and the ylide of *n*-heptyltriphenyl phosphonium bromide with hexanal gave 94% 6-tridecenes (*Z*:*E,* 95:5).

Chemical Analyses Lacewing extracts and chemical standards were analyzed in the splitless injection mode using an HP 6890 N gas chromatograph (GC) equipped with a DB-WaxETR column (0.25 μm film thickness, 30 m×0.25 mm ID; J & W Scientific, Folsom, CA, USA), with flame ionization detection. Hydrogen was used as the carrier gas, the injector and detector temperatures were 250°C and 300°C, respectively, and the column temperature was 50°C for 2 min, rising to 240°C at 10°C min^{-1}, and held for 10 min. GC-mass spectrometry (MS) was performed with an HP 6890 GC coupled to an HP 5973 mass selective detector using a DB-WaxETR column as above (except 60-m-long), programmed at 50°C for 2 min, rising to 230°C at 15°C min^{-1}, and held for 15 min.

Alkylthiolation for determining double bond position was accomplished using dimethyldisulfide (DMDS) derivatization as described by Zhu et al. (2000). For enhanced sensitivity in analyzing the reaction products, specific ion monitoring was used based on the *m/z* 61 ion $(CH_3SCH_2)^+$, which is present at 20–50% abundance in the spectra of all DMDS adducts (Leonhardt and DeVilbiss 1985).

Phylogenetic Analysis The Willi Hennig Society edition of "Tree analysis using New Technology" (TNT) software program was used to analyze the chemical data both qualitatively and quantitatively (Goloboff et al. 2008). Settings included a driven search for 500 replicates, set to find minimum length of one time per replicate. Search parameters included sectorial search, ratchet, drift, and tree fusing.

Results and Discussion

The results of our chemical analyses of lacewing PG secretions are summarized in Table 2. Including our analyses and those of others (Güsten and Dettner 1991; Zhu et al. 2000), the PG secretions of 23 species of green lacewings have now been analyzed, representing seven genera: those genera listed in Table 2, plus *Nineta* and *Chrysopidia* spp. (Güsten and Dettner 1991). Our results strongly support the conclusions of Güsten and Dettner (1991) that *(Z)*-4-tridecene is ubiquitous in green lacewing PG secretions, and other compounds are specific to one or only some genera, or some species within the *Chrysopa* (Table 2) as this genus now stands (Brooks and Barnard 1990; Brooks 1997).

We confirmed that the compound likely responsible for the fecal odor of *Chrysopa* spp. is skatole (3-methylindole) (Blum et al. 1973; Güsten and Dettner 1991), and extended the known distribution of this foul-smelling PG compound to two additional genera of green lacewings, *Plesiochrysa* and *Ceraeochrysa*. However, earlier reports of 1-tridecene in the prothoracic gland secretions of *Chrysopa oculata* (Blum et al. 1973; Zhang et al. 2004) and *Co. nigrispinus* (Zhang et al. 2006a) are here shown to be in error. Synthetic 1-tridecene did not coelute with the tridecene found in the PG secretions of these *Chrysopa* spp., whereas synthetic *(Z)*-4-tridecene did coelute with the natural products. The mass spectrum of 1-tridecene (not shown) exhibited a molecular ion (*m/z* 182) of about 15% the intensity of the base peak, whereas in the spectrum of *(Z)*-4-tridecene the molecular ion was nearly 60%. Furthermore, alkylthiolation of the prothoracic gland secretions of *Co. oculata* and *Co. nigrispinus* produced DMDS-derivatives showing the diagnostic mass spectral fragments for 4-tridecene (Zhu et al. 2000): m/z (%) 41(37), 55(47), 61(84), 69(86), 83(25), 103(65), 173(100), 229(4), and 276(M+, 21).

Beyond the universally present *(Z)*-4-tridecene, there are considerable differences in the distribution of other compounds among species, particularly for alkenes, alkadienes, and amides (Table 2). In three of the *Chrysopa* spp. we analyzed (*Co. nigrispinus*, *Co. incompleta*, and *Co. septempunctata*) PG secretions contained a 180 molecular weight

Table 2 Chrysopid prothoracic gland chemistry

Compound	Species[a] *P. r.*	*Co. n.*	*Co. i.*	*Co. s.*	*Co. o.*	*Co. q.*	*Ce. c.*	*Cl. r.*	*Cl.* sp.	*M.* sp.
(Z)-4-Undecene	40.17[b]	10.82	2.59					0.61		0.32
Undecadiene [c]	0.68									
(Z)-6-Tridecene		4.80	t [d]		t					
(Z)-4-Tridecene	1.35	26.33	40.39	13.98	62.10	53.63	19.90	89.49	100	90.39
(Z,Z)-4,7-Tridecadiene	26.42	27.99	22.65	20.86	3.50	t	1.49	9.49	t	5.59
Pentadecene [c]								0.19		0.35
Pentadecadiene [c]								0.22		t
Hexanoic acid							2.17			
Octanoic acid							1.04	t		
Nonanoic acid			1.48				0.66			
Skatole	16.92	23.17	24.87	32.28	22.29	40.37	49.78			
N-3-Methylbutylacetamide		6.44	6.05	32.86						
N-3-Methylbutylpropanamide	12.52	t	t							
3-hexanol							10.86			
2-hexanol							9.30			
Tridecanol	1.93									
Nonanal		t	3.52		7.00					
Decanal					5.10					
Benzaldehyde							4.82			

[a] *Plesiochrysa ramburi=P. r.*; *Chrysopa nigrispinus=Co. n.*; *Chrysopa incompleta=Co. i.*; *Chrysopa septempunctata=Co. s.*; *Chrysopa oculata= Co. o.*; *Chrysopa quadripunctata = Co. q.*; *Ceraeochrysa cubana = Ce. c.*; *Chrysoperla rufilabris=Cl. r.*; *Chrysoperla* sp.=*Cl.* sp.; *Mallada* sp.= *M.* sp.

[b] Percentages based on weighted mean gas chromatographic peak areas per compound per species.

[c] Standard not synthesized because insufficient natural product was available for further analysis; therefore, the identification is tentative based on mass spectral data; details in text.

[d] Trace≤0.1%.

compound, accounting for 20–30% of the excretion, which we thought to be a tridecadiene because the mass spectrum resembled that for *(Z)*-4-tridecene, but with the pattern of ions shifted two mass units lower. Indeed, the mass spectrum and retention time of the presumed lacewing tridecadiene matched the spectrum and retention time of synthetic *(Z,Z)*-4,7-tridecadiene, thus establishing the structure of the natural product. In *Plesiochrysa ramburi*, *(Z,Z)*-4,7-tridecadiene was much more abundant than *(Z)*-4-tridecene, and the eleven carbon alkene, *(Z)*-4-undecene [not 5-undecene as previously reported for *Co. nigrispinus* (Zhang et al. 2006a)], was identified as the major alkene constituent of the PG secretion based on mass spectral analysis of the DMDS derivative (Fig. 1A), and coinjection with the synthetic standard. The

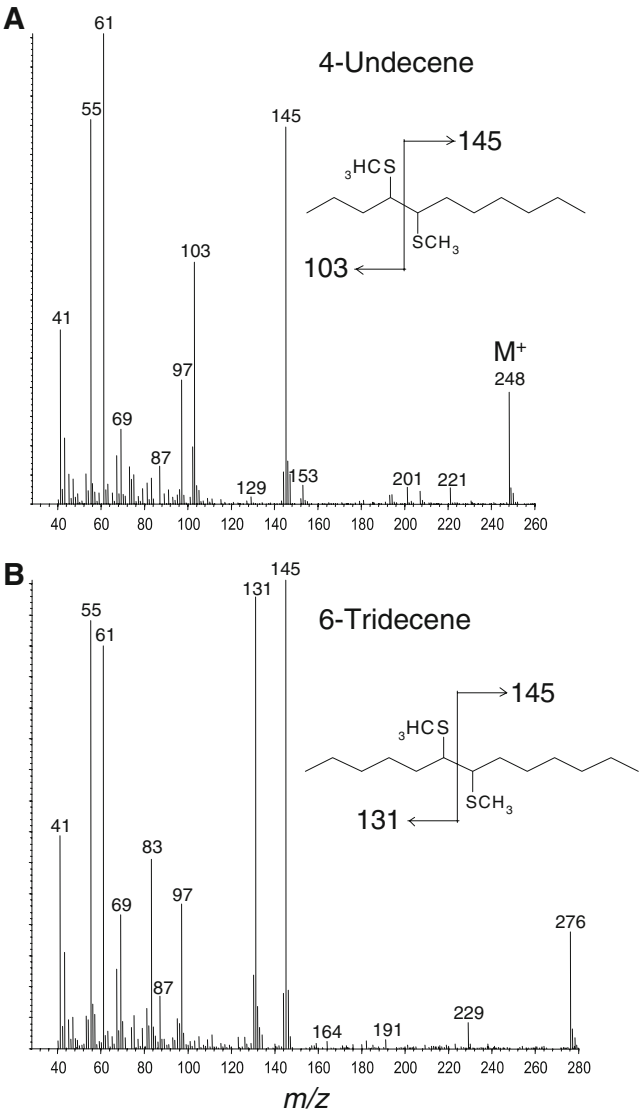

Fig. 1 Mass spectra of dimethyldisulfide derivatives of *(Z)*-4-undecene from the prothoracic gland (PG) secretion of *Plesiochrysa ramburi* (**A**), and *(Z)*-6-tridecene from the PG secretion of *Chrysopa nigricornis* (**B**)

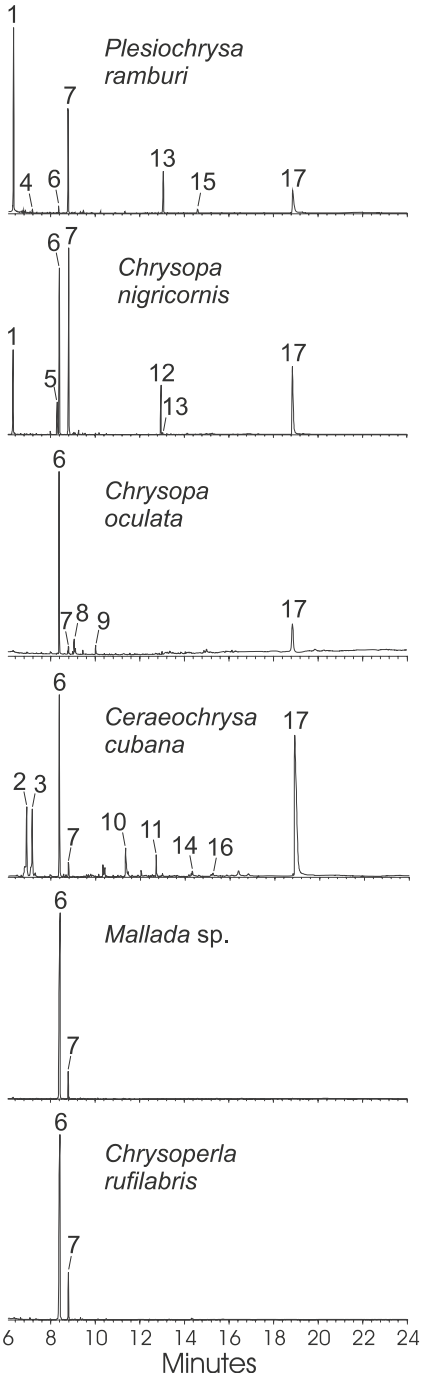

Fig. 2 Gas chromatograms of prothoracic gland extracts for representative species from the different genera and *Chrysopa* species groups analyzed [1=*(Z)*-4-undecene, 2=3-hexanol, 3=2-hexanol, 4=undecadiene, 5=*(Z)*-6-tridecene, 6=*(Z)*-4-tridecene, 7=*(Z,Z)*-4,7-tridecadiene, 8=nonanal, 9=decanal, 10=benzaldehyde, 11=hexanoic acid, 12=N-3-methybutylacetamide, 13=N-3-methylbutylpropanamide, 14=octanoic acid, 15=tridecanol, 16=nonanoic acid, and 17=skatole (3-methylindole)]

same group of *Chrysopa* spp. that express *(Z,Z)*-4,7-tridecadiene as a major PG secretion, also produced substantial quantities of N-3-methylbutylacetamide, a compound previously known as an allomone/pheromone in

cockroaches (Farine et al. 2002), yellowjacket wasps (Landolt and Heath 1987), and fruit flies (Bellas and Fletcher 1979). Curiously, the PG secretion of *P. ramburi* contained N-3-methylbutylpropanamide instead of the acetamide found in some of the *Chrysopa* species analyzed. Based on the mass spectrum of the DMDS derivative (Fig. 1B), as well as coinjection with the synthetic standard, *(Z)*-6-tridecene was also identified as a minor (*Co. nigricornis*) or trace (*Co. incompleta* and *Co. oculata*) PG component. Gas chromatograms of PG extracts for representative species from the different genera and *Chrysopa* species groups we examined are shown in Fig. 2.

Studies on pheromone biosynthesis in other insects suggest that *(Z)*-4-tridecene in lacewings may be biosynthesized from *(Z)*-9-octadecenoic (oleic) acid via successive cytosolic β-oxidation steps that remove four carbons, followed by decarboxylation (Jurenka 2004). *(Z)*-4-Undecene may be made from *(Z)*-9-hexadecenoic (palmitoleic) acid and tridecadiene most likely from *(Z,Z)*-9,12-octadecadienoic (linoleic) acid. Interestingly, *Chrysoperla carnea* is the only holometabolous insect known to synthesize linoleic acid (Cripps et al. 1986), and oleic and linoleic acids were the dominant fatty acids in abdominal extracts of *Co. oculata* in which fat body was present (JRA, unpublished data). Similarly, the biosynthesis of *(Z)*-6-tridecene may result from a single cycle of cytosolic β-oxidation of palmitoleic acid followed by decarboxylation.

The preliminary phylogenetic analysis of the semiochemical data (Table 2) resulted in an unresolved polytomy. Although the chemical signature of each of the species was distinct, the data set as a whole was more defined by autapomorphies than by synapomorphies. However, a grouping of *Chrysopa nigrispinus, Co. incompleta*, *and Co. septempunctata* was indicated based on the presence of N-3-methylbutylacetamide and the elevated expression of *(Z,Z)*-4,7-tridecadiene. Güsten and Dettner (1991) analyzed six *Chrysopa* species different from those we analyzed; all six of these *Chrysopa* were reportedly characterized by the presence of amides in their PG secretions (presumably the same acetamide we found). Thus, the *oculata-quadripunctata* grouping seems to represent a plesiomorphic clade distinct from those *Chrysopa* species that produce amides. Güsten and Dettner (1991) also noted that only *Chrysopa vididana* Schneider lacked skatole, which may be grounds to realign this species. Inclusion of other characters (e.g., morphological, molecular) in future analyses may provide further resolution.

Brooks and Barnard (1990) observed that "Chrysopidae is one of the largest and economically important families of the Neuroptera [~1200 species], [but] the classification of the family is confused." Much of the early chrysopid systematics was based on wing venation, which has been shown to be variable and unreliable (Brooks 1997). Today, quantitative computer analysis of DNA sequence data is the most powerful approach to deciphering phylogenetic relationships among insect taxa. Winterton and Freitas (2006) have applied this technique to the green lacewings, providing solid evidence that the Chrysopidae and Chrysopini are monophyletic, as well as clarifying the relationships of genera.

Apomorphic chrysopid PG semiochemicals are yet another set of quantifiable characters that may be incorporated with existing morphological, molecular, and courtship song data (Henry and Wells 2007) to further delineate relationships between green lacewings. Of course, many more species need to be analyzed chemically to lend strength to this approach. Preliminary efforts by one of us (JRA) have shown that it is feasible to obtain useful extracts of green lacewing PG secretions by briefly dipping live specimens in a minimal volume of solvent (unpublished data). At this point, the existing lacewing PG semiochemical data indicate that current *Chrysopa* spp. may be divisible based on the presence or absence of amides and skatole in their PG secretions. From a practical standpoint, the gas chromatographic "fingerprint" of chrysopid PG secretions (Fig. 2) appears to be a reliable means to help distinguish the common species of a region with the assistance of existing morphological keys (e.g., Penny et al. 2000). Certainly, one can quickly ascertain that a green lacewing that stinks is not a *Chrysoperla* species!

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