INTRA- AND INTERSPECIFIC COMMUNICATION IN THREE SPECIES OF *Glyptapanteles* PARASITIC WASPS (HYMENOPTERA: BRACONIDAE)

by

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ABSTRACT

Short-range pheromonal communication was investigated in congeneric *Glyptapanteles flavicoxis*, *G. indiensis* and *G. liparidis* (Hymenoptera: Braconidae). In coupled gas chromatographic-electroantennographic detection (GC-EAD) analyses of female *G. flavicoxis* body extracts, four components elicited strong responses from conspecific male antennae. Monitored by GC-EAD, the components were separated by flash silica gel and high-performance liquid chromatography. Y-tube olfactometer experiments revealed that all four components are necessary to elicit close-range attraction and wing-fanning responses by males.

In electrophysiological analyses of body extracts of female *G. indiensis* and *G. liparidis* conspecific male antennae responded to five and six components, respectively. Both species share four components with *G. flavicoxis*, but also have species-specific components. In Y-tube olfactometer experiments, body extracts of females elicited attraction and wing-fanning responses only by conspecific males, supporting the hypothesis of species-specific sex pheromone blends.

Keywords: *Glyptapanteles flavicoxis*, *Glyptapanteles indiensis*, *Glyptapanteles liparidis*, *Lymantria dispar*, *Lymantria obfuscata*, Hymenoptera, Braconidae, parasitoid, close-range sex pheromone, wing-fanning, species-specificity.

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1.1 Taxonomic relationships

Insects of the order Hymenoptera are taxonomically, biologically, and ecologically very diverse. Ants, bees, wasps, and sawflies represent the main groups of Hymenoptera. Most species are solitary, but some bee, ant, and wasp species exhibit high degrees of social organization. Some are phytophagous, whereas others are predatory or parasitic.

The Parasitica as a major division of the Hymenoptera comprises three superfamilies: Ichneumonoidea, Chalcidoidea, and Cynipoidea. The Ichneumonoidea are a dominant group divided in two families, the Ichneumonidae and Braconidae. At the family level, parasitoids exibit distinctive biological and behavioural characteristics. They attack host insects of all developmental stages (egg, larva, pupa, adult) from diverse orders (e.g., Homoptera, Coleoptera, Diptera, Lepidoptera, Hymenoptera). Adult parasitoids lay eggs on or within the host, and their developing larvae consume nutrients from the host, eventually killing it. Adult parasitoids are free-living, feeding on nectar, honeydew, or occasionally their host.

1.2 Sexual communication

Sexual communication is based on signal exchange between prospective mates (Matthews, 1975). Most conspicuous behavioural elements include orientation, attraction, recognition, wing-fanning, and antennation (Table 1.1). Females may attract males [e.g. *Syndipnus rubiginosus* (Ichneumonidae) (Eller et al., 1984)], or males may attract females [e.g. *Melittobia digitata* (Eulophidae) (Cônsoli et al., 2002)]. Males may wing fan only after they have made contact with females (Van den Assem, 1974), or not at all [*Diastrophus nebulosus* (Cynipidae) (Matthews, 1975)].

In some species, like *Aphytis melinus* (Aphelinidae), males engage in postcopulatory mate-guarding and courtship behaviour, attempting to prevent further matings of that female with other males. Such behaviour significantly increased the proportion of offspring the guarding male produced, and decreased significantly the female's chance of mating with another male (Allen et al., 1994). Male *Cephalonomia tarsalis* (Bethylidae) engage in aggressive precopulatory behavior that prevents rivals from mounting, or separates them from potential mates (Cheng et al., 2003).

1.3 Signals employed for sexual communication

Attraction or location of mates is mediated by specific sexual communication signals that are visual, pheromonal, sonic or tactile in nature. Here I will focus on pheromonal communication signals.

Table 1.1 Courtship behaviour in hymenopteran parasitoids

Species	Family	Biological characteristics	Host	Conspicuous behavioural elements during courtship	Reference
Aphytis spp.	Aphelinidae	Gregarious parasitoid	Homoptera: Diaspididae	Male: wing vibration; antennal contact with female's body	Rao & DeBach, 1969
Ascogaster reticulatus	Braconidae	Egg-larval solitary endoparasitoid	*Adoxophyes* sp., Lepidoptera: Tortricidae	Male: antennating substrate surface where a female walked; orienting to odour source by wing fanning	Kamano et al., 1989
Apanteles glomeratus	Braconidae	Gregarious larval parasitoid	*Pieris rapae crucivora,* Lepidoptera: Pyeridae	Male: orienting to female at close-range by visual cues; determining direction of the setae on the female's wings with his antennae; wing fanning	Tagawa, 1982
Cotesia rubecula	Braconidae	Solitary larval endoparasitoid	*Pieris rapae,* Lepidoptera: Pyeridae	Male: wing fanning, "pulsing" the abdomen, and transmitting vibrational signals through substrate Female: signalling receptivity by lowering antennae	Field & Keller, 1993
Cardiochiles nigriceps	Braconidae	Larval endoparasitoid	*Heliothis virescens,* Lepidoptera: Noctuidae	Male: wing fanning while orienting towards female's odour; antennal stroking of female's abdomen Female: signalling receptivity by raising antennae	Vinson, 1978
Diachasmimorpha kraussii	Braconidae	Solitary larval parasitoid	Diptera: Tephritidae	Male: wing-fanning Female: signalling receptivity by holding antennae back over the wings	Rungrojwanich & Walter, 2000
Aganaspis pelleranoi	Eucoilinae	Larval-pupal parasitoid	Diptera: Tephritidae	Male: wing fanning; antennal movements, contact with the female's body Female: signalling receptivity by holding antennae straight upward	Ovruski & Aluja, 2002

Table 1.1 continued

Species	Family	Biological characteristics	Host	Conspicuous behavioural elements during courtship	Reference
Melittobia chalybii, Melittobia megachilis	Eulophidae	Gregarious pupal ectoparasitoids	*Sarcophaga bullata,* Hymenoptera: Sphecidae	Male: wing raising; antennal contact with female's antennae Female: antennating the male's abdomen	Evans & Matthews, 1976
Campoletis sonorensis	Ichneumonidae	Gregarious larval endoparasitoid	*Heliothis virescens,* Lepidoptera: Noctuidae	Male: orienting to the odour source by wing fanning; antennal touching of female	Vinson, 1972
Megarhyssa sp.	Ichneumonidae	Larval parasitoid	*Tremex columba,* Hymenoptera: Siricidae	Males: forming aggregation on the bark of trees and competing for emerging females; bouts of wing vibration, "tergal stroking" - bending the abdominal tip and dragging it along substrate	Matthews et al., 1979
Lariophagus distinguendus	Pteromalidae	Larval-prepupal ectoparasitoid	Coleoptera	Male: wing fanning; after mounting antennal stroking of female's antennae Female: signalling receptivity by lowering head	Ruther et al., 2000
Nasonia vitripennis	Pteromalidae	Gregarious pupal parasitoid	cyclorrhaphous flies, Diptera	Male: wing fanning; "head-nodding" by extruding mouthparts and keeping them close to the female's antennae Female: signalling receptivity by lowering antennae	Miller & Tsao, 1974 Van den Assem et al., 1980

1.3.1 Mate-attracting sex pheromones

Mate-attracting sex pheromones are essential in the attraction and recognition of prospective mates, and have been reported in many species (Chapter 2; Table 1.2). They are typically released by females from various body parts (Table 1.3), and comprise components of different volatility that are effective at long- or short-range (Chapter 2; Table 1.4). Male-produced mate-attractant pheromones are rare among parasitic wasps. For instance, male *Melittobia digitata* (Eulophidae) develop, emerge, and mate within the cocoon of their host. They remain in the host cocoon, await the eclosion of their female siblings, and attract them with the pheromone α- and β-*trans*-bergamotene (Cônsoli et al., 2002).

1.3.2 Aggregation pheromones

Adults of *Brachymeria intermedia* and *B. lasus* (Chalcididae) overwinter in aggregations. While the aggregation pheromone has been identified as 3-hexanone in *B. intermedia*, the identity of the pheromone remains unknown in *B. lasus*. Such aggregations may increase the probability of mate location (Mohamed and Coppel, 1987b) and/or attract females to sites of high host densities (Kainoh, 1999).

1.3.3 Primer "aphrodisiac" pheromones

Male-produced aphrodisiac-type or primer pheromones that apparently enhance the females' receptivity have been reported in many parasitic wasps. They seem to be deployed in the antennation phase during which prospective mates make physical contact. Antennae of male *Leptomastix dactylopii*, *Rhopus meridionalis*, and *Asitus phragmitis* (all Encyrtidae) harbor pheromone glands. During complex courtship behaviour,

Table 1.2 Evidence for sex pheromones in hymenopteran parasitoids

Species	**Family**	**Biological characteristics**	**Host**	**Type of, and observational or experimental evidence for pheromone**	**Reference**
Aphelinus asychis	Aphelinidae	Nymphal solitary endoparasitoid	Homoptera: Aphididae	Females deposit pheromone on substrate	Fauvergue et al., 1995
Aphytis maculicornis, Aphytis mytilaspidis	Aphelinidae	Nymphal parasitoids	Homoptera: Aphididae	Female-produced sex-attractant pheromone; Male-produced close-range pheromone	Khasimuddin & DeBach, 1975
Aphidius colemani	Aphididae	Nymphal parasitoid	Homoptera: Aphididae	Female-produced sex attractant pheromone	Reed et al., 1994
Aphidius nigripes	Aphididae	Nymphal parasitoid	*Macrosiphum euphorbiae*, Homoptera: Aphididae	Female-produced sex attractant pheromone	McNeil & Brodeur, 1995
Apanteles glomeratus	Braconidae	Gregarious larval parasitoid	*Pieris rapae crucivora*, Lepidoptera: Pyeridae	Female-produced sex attractant pheromone	Obara & Kitano, 1974
Cotesia flavipes	Braconidae	Gregarious larval endoparasitoid	Lepidoptera	Female-produced sex-attractant pheromone; Male-produced sex-attractant pheromone	Kimani & Overholt, 1995
Diaeretiella rapae	Braconidae	Nymphal parasitoid	*Brevicoryne brassicae*, Homoptera: Aphididae	Female-produced sex-attractant pheromone	Askari & Alishah, 1979

Table 1.2 continued

Species	Family	Biological characteristics	Host	Type of, and observational or experimental evidence for pheromone	Reference
Opius alloeus	Braconidae	Pupal parasitoid	*Rhagoletis pomonella,* Diptera: Tephritidae	Female-produced sex-attractant pheromone	Mallory & Baerwald, 1967
Praon volucre	Braconidae	Nymphal parasitoid	Homoptera: Aphididae	Female-produced sex-attractant pheromone	Nazzi et al., 1996
Brachymeria intermedia	Chalcididae	Pupal endoparasitoid	*Lymantria dispar*, Lepidoptera: Lymantriidae	Female-produced sex-attractant pheromone; Female- and male-produced aggregation pheromone (3-hexanone)	Mohamed & Coppel, 1987a,b
Brachymeria lasus	Chalcididae	Pupal endoparasitoid	*Lymantria dispar*, Lepidoptera: Lymantriidae	Female-produced sex-attractant pheromone; Female- and male-produced aggregation pheromone	
Melittobia australica, Melittobia femorata	Eulophidae	Gregarious larval-pupal ectoparasioid	*Trypoxylon politum*, Hymenoptera: Sphecidae	Male-produced sex-attractant pheromone	Gonzalez et al., 1985
Campoletis sonorensis	Ichneumonidae	Gregarious larval endoparasitoid	*Heliothis virescens*, Lepidoptera: Noctuidae	Female-produced sex-attractant pheromone	Vinson, 1972
Ichneumon (= Pterocormus) promissorius	Ichneumonidae	Pupal endoparasioid	Lepidoptera: Noctuidae	Female-produced sex-attractant pheromone	Jewett & Carpenter, 1999
Anaphes listronoti	Mymaridae	Gregarious egg parasitoid	*Listronotus oregonensis*, Coleoptera: Curculionidae	Female-produced short and long-range sex-attractant pheromone	Cormier et al., 1998

Table 1.2 continued

Species	**Family**	**Biological characteristics**	**Host**	**Type of, and observational or experimental evidence for pheromone**	**Reference**
Lariophagus distinguendus	Pteromalidae	Larval-prepupal ectoparasitoid	Coleoptera	Female produced short-range sex-attractant pheromone; Paper discs treated with female extracts still active after several weeks	Ruther et al., 2000
Trichogramma brassicae	Trichogrammatidae	Egg parasitoid	Lepidoptera	Female-produced sex-attractant pheromone *plus* substrate-borne pheromone	Pompanon et al., 1997

Table 1.3 Source of pheromones in hymenopteran parasitoids

Species	Family	Biological characteristics	Host	Source of pheromone[1]	Reference
Aphidius nigripes	Aphididae	Nymphal parasitoid	*Macrosiphum euphorbidae*, Homoptera: Aphididae	Possibly abdomen	McNeil & Brodeur, 1995
Apanteles glomeratus	Braconidae	Gregarious larval parasitoid	*Pieris rapae crucivora*, Lepidoptera: Pyeridae	Abdomen at base of 2nd valvifer	Tagawa, 1977
Apanteles plutellae, *Apantele liparidis*, *Apanteles baoris*, *Apanteles ruficrus*, *Apanteles kariyai*	Braconidae	Larval parasitoids	Lepidoptera	Abdomen, glands on 2nd valvifers	Tagawa, 1983
Apanteles melanoscelus	Braconidae	Larval parasitoid	*Lymantria dispar*, Lepidoptera: Lymantriidae	Abdomen, epidermal glands on 8th abdominal tergum	Weseloh, 1976, 1980
Ascogaster reticulatus	Braconidae	Solitary egg-larval endoparasioid	*Adoxophyes* sp.; Lepidoptera: Tortricidae	Tibia of hindlegs	Kainoh & Oishi, 1993
Cardiochiles nigriceps	Braconidae	Larval endoparasitoid	*Heliothis virescens*, Lepidoptera: Noctuidae	Dufour's gland and cuticle	Syvertsen et al., 1995
Cotesia rubecula	Braconidae	Solitary larval endoparasitoid	*Pieris rapae*, Lepidoptera:	Abdomen near base of ovipositor	Field & Keller, 1994
Diaeretiella rapae	Braconidae	Nymphal parasitoid	*Brevicoryne brassicae*, Homoptera: Aphididae	Abdomen	Askari & Alishah, 1979
Macrocentrus grandii	Braconidae	Gregarious larval endoparasitoid	*Ostrinia nubilalis*, Lepidoptera: Pyralidae	During eclosion, mandibular glands; also in males	Swedenborg et al., 1993
Meteorus pulchricornis	Braconidae	Larval endoparasitoid	*Lymantria dispar*, Lepidoptera: Lymantriidae	Abdomen	Askari & Coppel, 1978

Table 1.3 continued

Species	Family	Biological characteristics	Host	Source of pheromone[1]	Reference
Praon volucre	Braconidae	Nymphal parasitoid	Homoptera: Aphididae	Abdomen	Nazzi et al., 1996
Campoletis sonorensis	Ichneumonidae	Gregarious larval endoparasitoid	*Heliothis virescens,* Lepidoptera: Noctuidae	By glands possibly associated with cuticle	Vinson, 1972
Melittobia australica Mellitobia femorata	Eulophidae	Gregarious pupal ectoparasitoids	*Trypoxylon politum,* Hymenoptera: Sphecidae	Males' abdomen	Gonzalez et al., 1985
Lariophagus distinguendus	Pteromalidae	Larval-prepupal ectoparasitoid	Coleoptera	Abdomen	Ruther et al., 2000
Nasonia vitripennis	Pteromalidae	Gregarious pupal parasitoid	cyclorrhaphous flies, Diptera	Possibly abdomen	Van den Assem et al., 1980
Roptrocerus xylophagorum	Pteromalidae	Larval-pupal ectoparasitoid	Coleoptera: Scolytidae	Entire body surface	Sullivan, 2002

[1]In females unless otherwise stated

Table 1.4 Sex pheromone components (tentatively) identified in hymenopteran parasitoids

Species	Family	Biological characteristics	Host	Sex pheromone components[1]	Reference
Ascogaster reticulatus	Braconidae	Solitary egg-larval endoparasioid	*Adoxophyes* sp., Lepidoptera: Tortricidae	(*Z*)-9-hexadecenal	Kainoh et al., 1991 Hidoh et al., 1992
Ascogaster quadridentata	Braconidae	Solitary egg-larval endoparasitoid	*Cydia pomonella*, Lepidoptera: Tortricidae	(*Z*,*Z*)-9,12-octadecadienal, (*Z*)-9-hexadecenal, 3,7,11-trimethyl-6E,10-dodecadiennal	DeLury et al., 1999
Cardiochiles nigriceps	Braconidae	Larval endoparasitoid	*Heliothis virescens*, Lepidoptera: Noctuidae	(*Z*,*Z*)-7,13-heptacosadiene *plus* other unknown hydrocarbons	Syvertsen et al., 1995
Macrocentrus grandii	Braconidae	Gregarious larval endoparasitoid	*Ostrinia nubilalis*, Lepidoptera: Pyralidae	(*Z*)-4-tridecenal, (3*R*,5*S*,6*R*)-3,5-dimethyl-6-(methylethyl)-3,4,5,6-tetrahydropyran-2-one, (*Z*,*Z*)-9,13-heptacosadiene	Swedenborg & Jones, 1992 a,b; Swedenborg et al., 1993
Melittobia digitata	Eulophidae	Gregarious larval -pupal ectoparasioid	Diptera	Male-produced α- and β-*trans*-bergamotene	Cônsoli et al., 2002
Eriborus terebrans	Ichneumonidae	Larval parasitoid	*Ostrinia nubilalis*, Lepidoptera: Pyralidae	Polar component with properties of a carboxylic acid *plus* additional oxygen-containing functional group, hydrocarbon(s)	Shu & Jones, 1993
Itoplectis conquisitor	Ichneumonidae	Pupal endoparasitoid	Lepidoptera	Neral and/or geranial components	Robacker & Hendry, 1977
Syndipnus rubiginosus	Ichneumonidae	Larval endoparasitoid	*Pikonema alaskensis*, Hymenoptera: Tenthredinidae	Ethyl (*Z*)-9-hexadecenoate	Eller et al., 1984
Roptrocerus xylophagorum	Pteromalidae	Larval-pupal ectoparasitoid	Coleoptera: Scolytidae	Cuticular hydrocarbon alkanes	Sullivan, 2002

[1] Produced by females unless otherwise stated

the males deposit secretions from these glands onto the females' antennae, which in turn, elicit the males' acceptance by females (Guerrieri et al., 2001).

Similarly, antennae of *Amitus spiniferus* (Platygastridae) have a paddle-shaped "sex-male" segment which secrets a mate-recognition and/or aphrodisiac pheromone onto the females' antennae during courtship (Isidoro and Bin, 1995). Such secretory glands with various pheromone-releasing structures on different antennomeres have also been reported in eulophid (Dahms, 1984), scelionid (Bin and Vinson, 1986), and aphelinid (Pedata et al., 1995) parasitoids.

Intermediate antennal segments of male *Pimpla turionellae* (Ichneumonidae) have a callous-type appearance (tyloid), which secret pheromone during the antennation phase of courtship. Intriguingly, the males' intensity of antennal stroking is dependent on the females' receptivity (Bin et al., 1999).

1.4 Life history of *Glyptapanteles flavicoxis*, *G. indiensis*, and *G. liparidis*

Information about the biology of *G. flavicoxis* (Braconidae) is scarce. *Glyptapanteles flavicoxis* is a gregarious, koinobiont endoparasitoid of larval Indian gypsy moth, *Lymantria obfuscata* (Lepidoptera: Lymantriidae) (Marsh, 1979). In 1981, it was imported from India and released into North America as a potential biological control agent for larvae of the European gypsy moth, *Lymantria dispar* (Lepidoptera: Lymantriidae) (Krause et al., 1990, 1991). Single *G. flavicoxis* cocoons are found on early instar *L. obfuscata* larvae, whereas clusters of cocoons are found on late instar host larvae. Adult *G. flavicoxis* are active from April through July. There are possibly four generations per year, each requiring 17-35 days for completion (Krause, 1987).

Laboratory experiments have demonstrated that *G. flavicoxis* develop in all *L. dispar* instars, but females attack significantly more early (2^{nd} & 3^{rd}) than late (4^{th}-6^{th}) instars (Fuester et al., 1987). Pro-ovigenic females carrying 200-250 eggs commonly oviposit more than one egg into a host larva (Krause et al., 1991). Parasitoid larvae develop inside the larval host, allowing it to continue to live until they exit it. After about 2-3 weeks of development, few to several hundred parasitoid larvae emerge from, and pupate around, the host in characteristic clusters of whitish cocoons. During the 1^{st} week of the pupal period cocoons become hard and black. Females generally are larger and develop more slowly than males (Krause, 1987).

Similar to other arrhenotokous parasitoids, *G. flavicoxis* has a haplo-diploid mechanism of sex determination; fertilized eggs give rise to female progeny, whereas unfertilized eggs give rise exclusively to male progeny. The sex ratio is male-biased (4:1) (Krause, 1987).

Sympatric *G. indiensis* is a solitary parasitoid of 1^{st} instar *L. obfuscata*, which occurs in northern parts of India, Pakistan and Afghanistan. A single parasitoid larva develops inside a host larva and pupates in a whitish cocoon away from the host.

Allopatric *G. liparidis* occurs in Japan, Korea, the Kurile Islands, Russia, North Africa, and Europe (Marsh, 1979). It is a multivoltine braconid with 4 generations per year, attacking 2^{nd} and 3^{rd} instars of *L. dispar,* and alternate host species, including *Dendrolimus* spp. (Lepidoptera: Lasiocampidae), its primary overwintering host. As a gregarious endoparasitoid, female *G. liparidis* may lay up to 100 eggs in a single host. Parasitoid larvae spin their whitish cocoons in an irregular cluster mostly away from the host. The developmental time for the egg-larval and pupal stage is 25-35 days, and 6-8

days, respectively. Adult females live about 14 days, and males 10 days (Houping and Jingjun, 1993).

1.5 Current knowledge about pheromonal communication in *G. flavicoxis*, *G. indiensis*, and *G. liparidis*

Female *G. flavicoxis* press their abdominal tip to the substrate, apparently depositing pheromone that elicits wing-fanning by males (Fuest[1], personal communication). This interpretation of the females' behaviour is supported by reports of abdominal pheromone glands in other braconid females, including *Ap. glomeratus* (Tagawa, 1977), *Ap. melanoscelus* (Weseloh, 1976, 1980), *Ap. plutellae, Ap. liparidis, Ap. baoris, Ap. ruficrus*, and *Ap. kariyai* (Tagawa, 1983). Female *G. flavicoxis* also employ an airborne component (ethyl dodecanoate) which by itself is not effective in attracting conspecific males (Fuest, personal communication).

Pheromonal communication of *G. liparidis* and *G. indiensis* has not yet been investigated, but one might speculate that it is similar to that of *G. flavicoxis*. As congeners, they may share pheromone components, while using species-specific components to enhance reproductive isolation, particularly when they occur in sympatry.

[1] Jamie Fuest, former undergraduate research assistant in Gries-laboratory, unpublished observation.

1.6 Research objectives

My research objectives were:

1. to investigate whether female *G. flavicoxis* use sex pheromone components, and, if so, to isolate them and determine their behavioural role; and

2. to test the hypothesis that *G. flavicoxis*, *G. indiensis*, and *G. liparidis* use species-specific sex pheromone components to confer specificity to their sexual communication systems.

2 EVIDENCE FOR 4-COMPONENT CLOSE-RANGE SEX PHEROMONE IN *G. flavicoxis*

2.1 Introduction

Sex pheromones in hymenopteran parasitic wasps are typically produced by females. They have been reported in seven families [Aphelinidae, Chalcididae, Cynipidae, Pteromalidae, Scelionidae, Braconidae and Ichneumonidae (Kainoh, 1999)], but have been identified only in a few species, including *Itoplectis conquisitor* (Ichneumonidae) (Robacker and Hendry, 1977), *Syndipnus rubiginosus* (Ichneumonidae) (Eller et al., 1984), *Macrocentrus grandii* (Braconidae) (Swedenborg and Jones, 1992a,b, 1993), *Ascogaster reticulatus* (Braconidae) (Kainoh et al., 1991), *Cardiochiles nigriceps* (Braconidae) (Syvertsen et al., 1995), and *As. quadridentata* (Braconidae) (DeLury et al., 1999).

In the Braconidae, sex pheromones have been reported, but not identified, in *Opius alloeus* (Boush and Baerwald, 1967), *Apanteles medicaginis* (Cole, 1970), *Ap. glomeratus* (Obara and Kitano, 1974), *Ap. melanoscelus* (Weseloh, 1976, 1980), *Cotesia rubecula* (Field and Keller, 1994), *C. flavipes* (Kimani and Overholt, 1995), *Praon volucre* (Nazzi et al., 1996), and *Fopius arisanus* (Quimio and Walter, 2000). Most are long-range attractants.

Substrate-borne sex pheromones in parasitoids are rare. Female *Aphelinus asychis* (Aphelinidae) appear to have a trail pheromone, but do not exhibit specific trail marking behaviour (Fauvergue et al., 1995). In *Trichogramma brassicae* (Trichogrammatidae), a

substrate-borne pheromone induces male searching in an area previously explored by females, and attracts males from short-distance (Pompanon et al., 1997). Female *As. reticulates*, egg-larval parasitoids of the smaller tea tortrix, *Adoxophyes* sp., employ short-range pheromones that activate searching by males and increase the probability of mating (Kamano et al., 1989).

Some parasitic wasps have multiple-component pheromones. For example, male *M. grandii* are attracted to the female-produced components (*Z*)-4-tridecenal and (*Z,Z*)-9,13-heptacosadiene (Swedenborg and Jones, 1992a,b). The behavioural activity of both compounds is enhanced by (3*R*,5*S*,6*R*)-3,5-dimethyl-6-(methylethyl)-3,4,5,6-tetrahydropyran-2-one as a third component that is biosynthesized in mandibular glands of both males and females (Swedenborg et al., 1993). In the ichneumonid *Eriborus terebrans*, the nonpolar pheromone component by itself is inactive, but when added to the polar component provokes the male's behavioural response (Shu and Jones, 1993).

My objective was to investigate whether female *G. flavicoxis* use sex pheromone components, and, if so, to isolate them and determine their behavioural role.

2.2 Methods and materials

2.2.1 Experimental insects

The rearing colonies of experimental insects in the Global Forest Quarantine Facility at Simon Fraser University (SFU) were started and augmented with specimens obtained from the Beneficial Insects Introduction Research Laboratory, United States Department of Agriculture, Agricultural Research Service, Newark, Delaware. To facilitate mating in *G. flavicoxis*, 10 females and 30 males were placed in plastic mesh

cages (10 x 10 x 6 cm) (Hu et al., 1986), and provisioned with cotton wicks (1 x 10 cm; Richmond Dental, Charlotte, North Carolina) soaked in sugar water solution. Oviposition cages (18 x 18 x 12 cm) contained 10-15 mated females, five *L. dispar* larvae (3-4 instar) (Fuester et al., 1987), and artificial diet for the larvae (Bell et al., 1981). After 1-2 days, parasitized host larvae were removed and placed on artificial diet in plastic cups (192 ml) with tight-fitting paper lids (Sweetheart Plastics, Wilmington, Massachusetts). Every second day, larval frass was removed, diet replenished if needed, and parasitoid cocoons with insects to be used in bioassays were transferred individually to capped plastic cups (30 ml) provisioned with sugar water-soaked cotton wicks. Cocoons of insects to be used for mass rearing were placed in plastic Petri dishes (14 cm diam.) and food-provisioned as described above. Rearing took place under a 16L:8D photoregime at 22-25 °C and 50-70% RH.

2.2.2 Acquisition of volatiles

Unmated, 1- to 2-day-old females (5-10) were placed into vertical cylindrical Pyrex glass chambers (6 x 10 cm ID), and were provisioned with a sugar water-soaked cotton wick. Control chambers contained the same food source, but no parasitoids. A water aspirator drew humidified, charcoal-filtered air at a rate of 1.5-2 L/min for 2 days through the chambers and a glass column (14 x 1.3 cm OD) filled with 150 mg of Porapak Q (50-80 mesh, Waters Associates Inc., Milford, Massachusetts, USA). Volatiles were eluted from the Porapak Q volatile traps with redistilled pentane (2 ml). The extract was concentrated under a stream of nitrogen such that 10 µl of extract contained one female hour equivalent (FHE) of volatile acquisition (= amount of volatiles released by 1 female during 1 hour).

2.2.3 Acquisition of pheromone extracts

Females (1-3 days old) were macerated in vials containing hexane (ca. 10 µl per female) placed on dry ice. Then the extract was kept at room temperature for ~ 15 min. The supernatant was withdrawn, filtered through a small amount of glass wool in a pipette, and quantified to determine the volume representing one female body extract equivalent (FBE).

2.2.4 Video-recording of trail-following behaviour by males

To test the hypothesis that males follow a pheromone trail, their behavioural response was video-recorded (Sony Digital Video Camera Recorder, DCR-VX 1000). In each of 10 Pyrex glass dishes (9 x 2 cm high), 1 FBE was pipetted in trail-like pattern (Figure 2.1). Additional 10 Pyrex glass dishes served as a control stimulus, with solvent applied in the same way as the treatment stimulus. After the solvent had evaporated (~10 sec), a virgin 1- to 3-day-old male was released and video-recorded for 5 min. Recordings were analyzed for the time a male had spent on the trail and for other characteristic behavioural responses, such as wing-fanning.

2.2.5 Y-tube olfactometer bioassays

All experiments were conducted during hours 2 to 6 of the insects' photophase (16L:8D). Anemotactic responses of males to odour sources were tested in vertical Pyrex glass Y-shaped olfactometers (stem: 20 x 2.5 cm ID; side arms at 120°: 18 cm long) positioned vertically 15 cm below a light source, consisting of one tube of fluorescent "daylight" (F40DX, H118; Osram Sylvania Ltd., Ontario, Canada) and one tube of "wide spectrum grow light" (F40GRO/WS, H658; Osram Sylvania Ltd., Ontario, Canada).

Treatment or control (solvent) stimuli were pipetted on white strips of paper (15 x 1 cm) placed in side arms of the Y-tube (Experiments 1-28), or on filter paper discs (4.3 cm diam., Whatman No. 1, Whatman International Ltd. Maidstone, England) placed near the orifice of side arms (Experiments 29, 30).

In experiment 31, two live 2- to 3-day-old females served as a test stimulus. They were transferred 10-15 min before experimental replicates into mesh-covered glass tubes (6 x 2 cm ID), and provisioned with a sugar water-soaked cotton wick. Treatment and control tubes (lacking females) were placed at the orifice of side arms of the Y-tube olfactometers.

In all experiments, a water aspirator drew air at ~ 1 L/min through the Y-tube to test anemotactic responses of parasitoids released individually into the stem of the Y-tube. An insect was classed a responder when it traversed the entire paper strip up to the orifice of the side arm (Experiments 1-28), or contacted the filter paper discs (Experiments 29, 30), or glass tube housing two females (Experiment 31) within 10 min. All others were classified as non-responders. For each replicate, a new insect, paper strip, filter paper disc, and clean (Sparkleen-washed and oven-dried) Y-tube, or glass tube, were used, with test stimuli randomly assigned to side arms.

To compare the attractiveness of test stimuli most rigorously, two to four experiments were often run in parallel over 2-4 days, alternating between replicates for each experiment. To gauge the relative attractiveness of two or more test stimuli, parallel experiments proved to be more effective than head-to-head comparisons of stimuli in the same Y-tube olfactometer. The number of parasitoids responding to stimuli were analysed with the χ^2 goodness-of-fit test using Yates' correction for continuity (α =

0.05), testing the null hypothesis that insects did not prefer either treatment or control stimuli (Zar, 1996).

Experiment 1 tested the "trail-following" response by males. Experiments 2 and 3 determined whether the females' body extract in combination with the females' effluvia, or synthetic effluvium component ethyl dodecanoate, were similarly effective in attracting males. Experiments 4 and 5 determined whether males or females respond to the pheromone. Experiments 6-8 explored the relative attractiveness of body extract, ethyl dodecanoate, or both.

Experiments 9-12 tested whether silica fraction 4 (containing candidate close-range pheromone components) and female body extract (containing candidate close-range pheromone components plus traces of ethyl dodecanoate and possibly other components) were equally attractive, at a low dose (1 FHE plus 1 FBE) or medium dose (5 FHE plus 5 FBE). Taking into account that silica fraction 4, at the medium dose, was very effective in attracting males, experiments 13 and 14 re-tested whether ethyl dodecanoate enhances the attractiveness of silica fraction 4. Although ethyl dodecanoate did not seem critical for attraction of males, it was retained in subsequent experiments (15-27, 30) to ensure the best possible response of males to all test stimuli, and to allow the best comparison of results in all experiments.

Experiments 15 and 16 tested silica fraction 4 at the medium dose versus the combination of all HPLC fractions that contained candidate close-range pheromone components (= effective blend). Considering the strong attractiveness of the effective blend, follow-up experiments 17, 19, 21 and 23 explored whether one or more of the candidate close-range pheromone components 1 (HPLC fractions 25-28), 2 and 4 (HPLC

fractions 21-24), or 3 (HPLC fractions 16-20) could be deleted from the effective blend without affecting the males' attraction or wing-fanning response. Experiments 25-27 tested the males' attraction and wing-fanning responses to ethyl dodecanoate alone (Experiment 27), or in combination with either the effective blend (Experiment 25) or most EAD-active pheromone component 3 (Experiment 26).

Placement of test stimuli near (~1 cm) the junction of Y-tubes in experiments 1-28 was appropriate to test close-range anemotactic and wing-fanning responses of males, but not very suitable to determine whether ethyl dodecanoate, or other female-produced components, might enhance the active space (mate-recruiting distance) of the entire pheromone blend. Thus, final experiments 29-31 tested the response of males to stimuli [silica fraction 4 on filter paper disc (Experiment 29); silica fraction 4 plus ethyl dodecanoate on filter paper disc (Experiment 30); 2 caged live females (Experiment 31)] that were placed at the orifice of side arms >10 cm apart from the junction of the Y-tube.

2.2.6 Analyses of pheromone extracts

Aliquots of 1 FHE or 1 FBE were analyzed by coupled gas chromatographic-electroantennographic detection (GC-EAD) (Arn et al., 1975; Gries et al., 2002a), employing a Hewlett Packard (HP) 5890A gas chromatograph equipped with a GC column (30 m x 0.25 or 0.32 mm ID) coated with DB-5, DB-17, DB-210, DB-23 or FFAP (J & W Scientific, Folsom, California 95630). For GC-EAD recordings, a male's head was severed and placed into the opening of a glass capillary electrode filled with saline solution (Staddon and Everton, 1980). One antenna with its tip removed by spring microscissors (Fine Science Tools Inc., North Vancouver, British Columbia, Canada) was placed into the opening of a second (indifferent) electrode.

EAD-active compounds were analyzed by (1) full-scan electron-impact and chemical ionization (CI, acetonitrile) mass spectrometry (MS) with a Varian Saturn 2000 Ion Trap GC-MS fitted with the DB-5 column referred to above; (2) retention index calculations (Van den Dool and Kratz, 1963); and (3) microanalytical treatments (hydrogenation, oxidation, reduction, acetylation, deacetylation) followed by renewed GC-EAD and GC-MS of the extract.

Aliquots of 100 FBEs with EAD-active components were fractionated through silica gel (0.5 g) in a glass column (14 x 0.5 cm ID). After the silica was pre-rinsed with pentane, the extract was applied, allowed to impregnate the silica gel, and then eluted with six consecutive rinses (1 ml each) of pentane/ether, with increasing proportion of ether, as follows: (1) 100:0; (2) 100:0; (3) 90:10; (4) 75:25; (5) 50:50 and (6) 0:100. This procedure generated fractions containing analytes of increasing polarity.

To determine silica fractions with candidate pheromone components, fractions were concentrated to the corresponding number of female equivalents processed in the initial extract, and analysed by GC-EAD, co-injecting as an internal standard ethyl dodecanoate (1 ng), which eluted 4-8 min earlier on the different GC columns than any of the four components. Fractions with more than one EAD-active compound (= candidate pheromone component) were fractionated further into 40 fractions (1 fraction / 25 sec) by high-performance liquid chromatography (HPLC), followed by renewed GC-EAD analyses of all HPLC fractions. HPLC fractionation employed a Waters LC 626 HPLC equipped with a Waters 486 variable wavelength UV visible detector set to 210 nm, HP Chemstation software (Rev. A.07.01), and a reverse-phase Nova-Pak C18 column (60 Å, 4 μm; 3.9 x 300 mm) eluted with 1 ml/min of 100% acetonitrile.

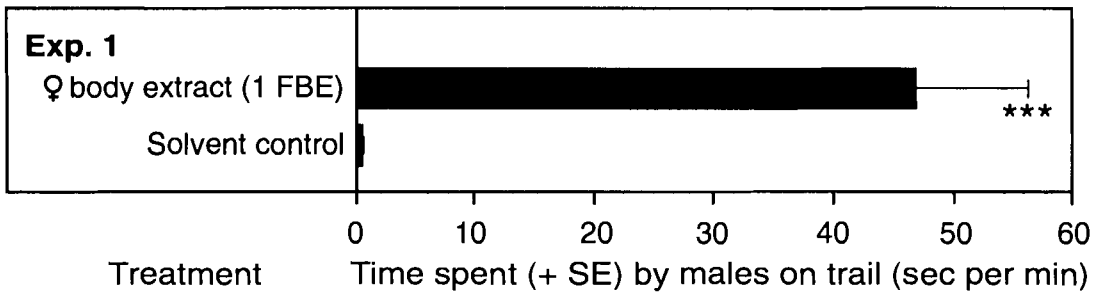
2.3 Results

In experiment 1, 1 FBE induced wing-fanning and "trail-following behaviour" by males (Figure 2.1). Males also spent significantly more time on trails of body extract of females than on solvent control trails (Figure 2.1).

Effluvium (1 FHE) and body extract (1 FBE) of females in combination attracted significantly more males than did the solvent control (Figure 2.2, Experiment 2). Similarly, ethyl dodecanoate plus female body extract significantly attracted males (Figure 2.2, Experiments 3, 5 and 8), but not females (Figure 2.2, Experiment 4). Unlike female body extract, ethyl dodecanoate by itself failed to significantly attract males or to provoke wing-fanning (Figure 2.2, Experiments 6, 7).

GC-EAD analyses of female body extracts revealed four components that elicited antennal responses from males (Figure 2.3; Table 2.1). Although these components appeared to be abundant in the corresponding FID trace, their mass spectra suggested that they were not pheromone components, but superimposed on them. GC-EAD analyses of all six silica fractions of female body extract revealed that fraction 4 contained the four EAD-active components, and that they indeed occurred below FID detection threshold (Figure 2.3). In Y-tube olfactometers, female body extract (at 1 FBE) combined with ethyl dodecanoate was attractive, whereas silica fraction 4 (at 1 FBE) with ethyl dodecanoate was not (Figure 2.4, Experiments 9, 10), suggesting that some active material had been lost during fractionation. However, silica fraction 4 at 5 FBE together with ethyl dodecanoate, significantly attracted males (Figure 2.4, Experiment 11), indicating that all essential components of the close-range pheromone were present in silica fraction 4.

Figure 2.1 *(Top)* Time spent by male *Glyptapanteles flavicoxis* (n = 10) on line drawings of a trail treated with one female body extract equivalent (1 FBE) or a solvent control. Single-factor analysis of variance, $P < 0.05$; *(Bottom)* Representative example of "trail-following behaviour" by a male (depicted as arrow head), with the position recorded every 2 sec.


Exp. 1
♀ body extract (1 FBE)
Solvent control
***
0
10
20
30
40
50
60
Treatment
Time spent (+ SE) by males on trail (sec per min)

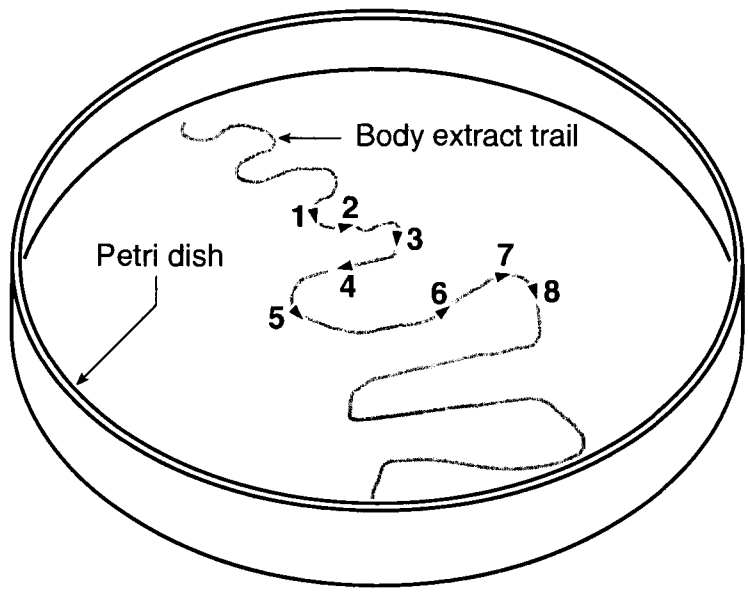

Body extract trail
Petri dish
1
2
3
4
5
6
7
8

Figure 2.2 Number of male or female *Glyptapanteles flavicoxis* that were attracted, or wing-fanned, in response to test stimuli tested in Y-tube olfactometer experiments 2-8. 1 FHE = one female hour equivalent = pheromone component(s) released by one female during one hr; 1 FBE = one female body extract equivalent = pheromone component(s) contained in extract of one macerated female body. In each experiment, bars with asterisks indicate a significant response to a particular treatment; χ^2 test (Experiments 2 and 3), heterogeneity χ^2 test with Yates' correction for continuity, treatment versus control; *P < 0.05; **P < 0.01; ***P < 0.001. Note: (1) Experiments grouped by brackets were run in parallel; (2) only after completion of experiment 3 did I realize that I should have recorded wing-fanning as a response criterion.

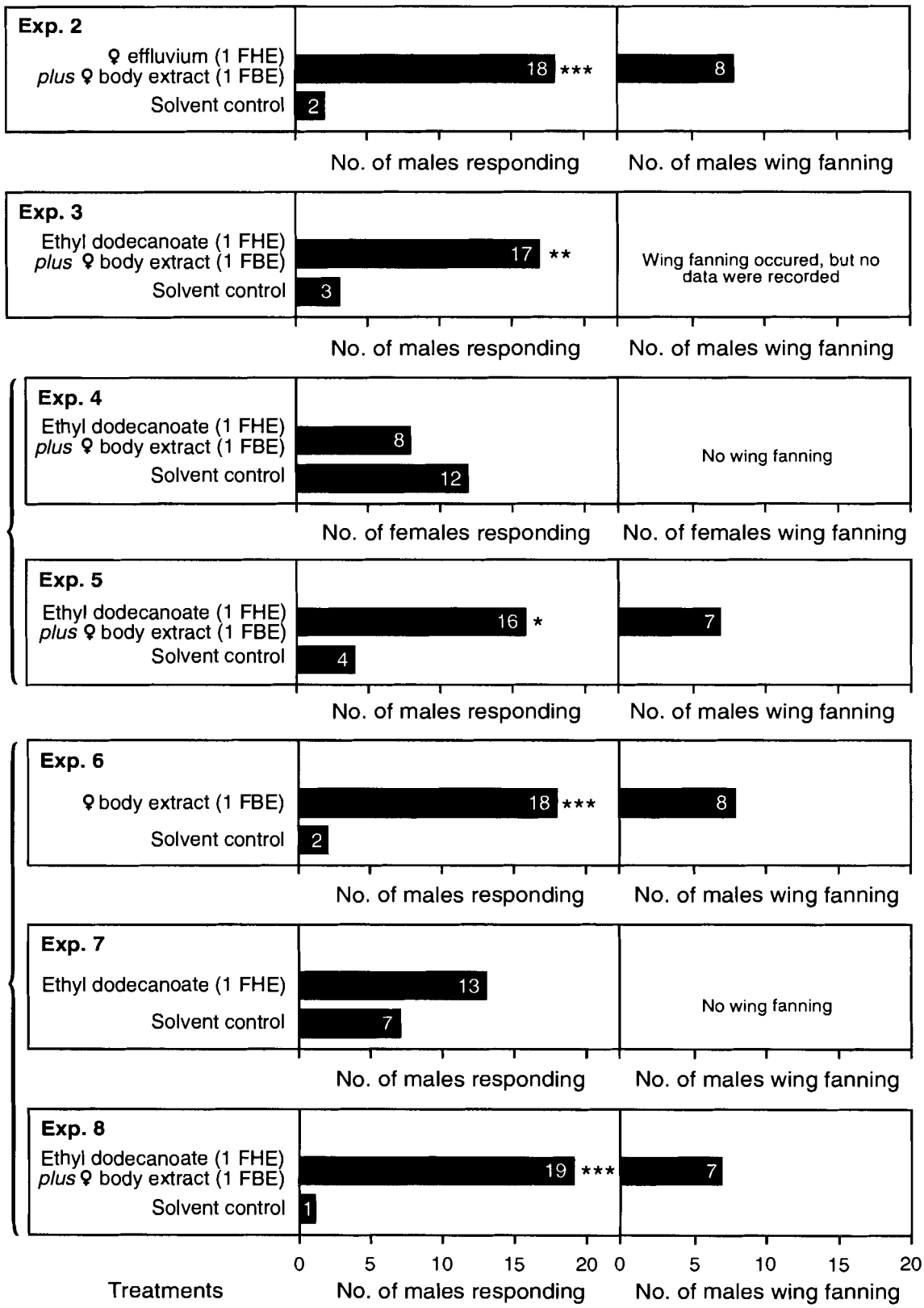

Exp. 2
♀ effluvium (1 FHE) plus ♀ body extract (1 FBE)
18 ***
8
Solvent control
2
No. of males responding
No. of males wing fanning
Exp. 3
Ethyl dodecanoate (1 FHE) plus ♀ body extract (1 FBE)
17 **
Wing fanning occured, but no data were recorded
Solvent control
3
No. of males responding
No. of males wing fanning
Exp. 4
Ethyl dodecanoate (1 FHE) plus ♀ body extract (1 FBE)
8
No wing fanning
Solvent control
12
No. of females responding
No. of females wing fanning
Exp. 5
Ethyl dodecanoate (1 FHE) plus ♀ body extract (1 FBE)
16 *
7
Solvent control
4
No. of males responding
No. of males wing fanning
Exp. 6
♀ body extract (1 FBE)
18 ***
8
Solvent control
2
No. of males responding
No. of males wing fanning
Exp. 7
Ethyl dodecanoate (1 FHE)
13
No wing fanning
Solvent control
7
No. of males responding
No. of males wing fanning
Exp. 8
Ethyl dodecanoate (1 FHE) plus ♀ body extract (1 FBE)
19 ***
7
Solvent control
1
0 5 10 15 20
0 5 10 15 20
Treatments
No. of males responding
No. of males wing fanning

Figure 2.3 Flame ionization detector (FID) and electroantennographic detector (EAD: male *Glyptapanteles flavicoxis* antenna) responses to aliquots of female body extract (*top*), silica fraction 4 (*middle*), and HPLC fractions 16-20, 21-24 and 25-28 (*bottom*). Chromatography: Hewlett Packard 5890A equipped with a DB-23 coated column (30 m x 0.25 mm ID); linear flow velocity of carrier gas: 35 cm/sec; injector and FID detector temperature: 220°C; temperature program: 1 min at 100°C, 10°C/min to 220°C.

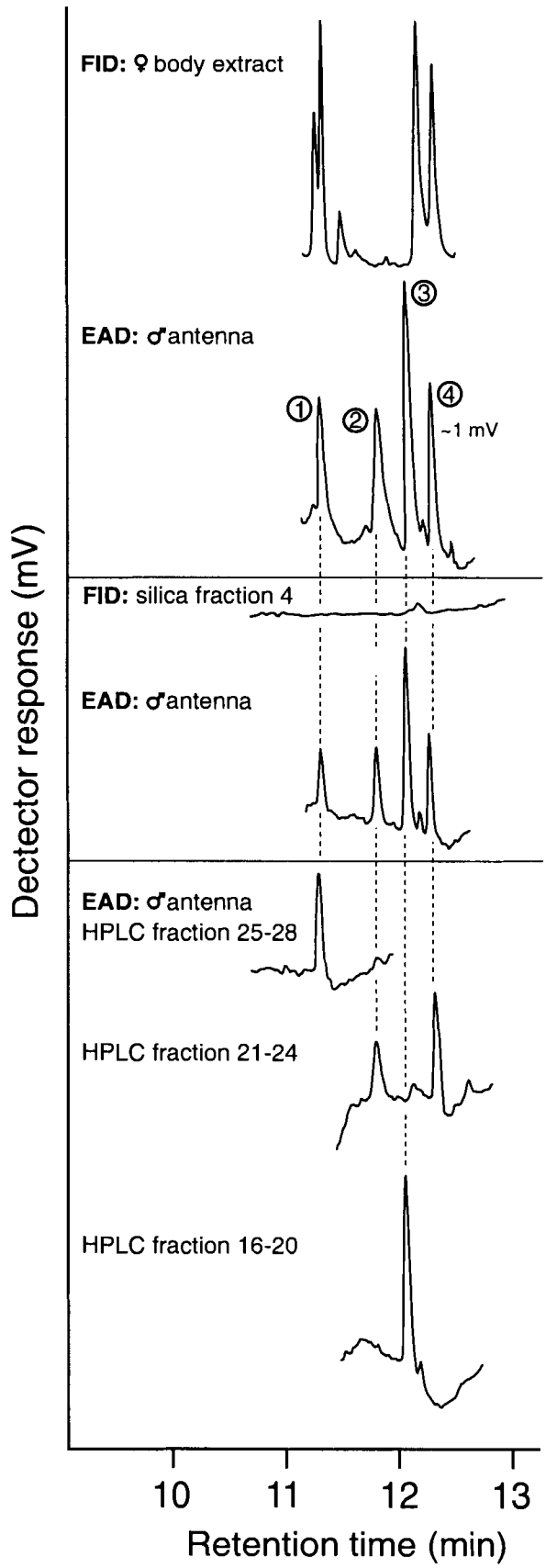

FID: ♀ body extract
EAD: ♂ antenna
①
②
③
④
~1 mV
FID: silica fraction 4
EAD: ♂ antenna
EAD: ♂ antenna
HPLC fraction 25-28
HPLC fraction 21-24
HPLC fraction 16-20
Dectector response (mV)
10
11
12
13
Retention time (min)

Table 2.1 Retention indices (relative to alkane standards) (Van den Dool and Kratz, 1963) of pheromone components 1-4 in body extracts of female *Glyptapanteles flavicoxis* (Figure 2.3), and ability of microanalytical treatments of silica or HPLC fractions of body extracts to alter the molecular structure of components 1-4, as determined by the presence or absence of respective antennal responses in GC-EAD recordings of such fractions.

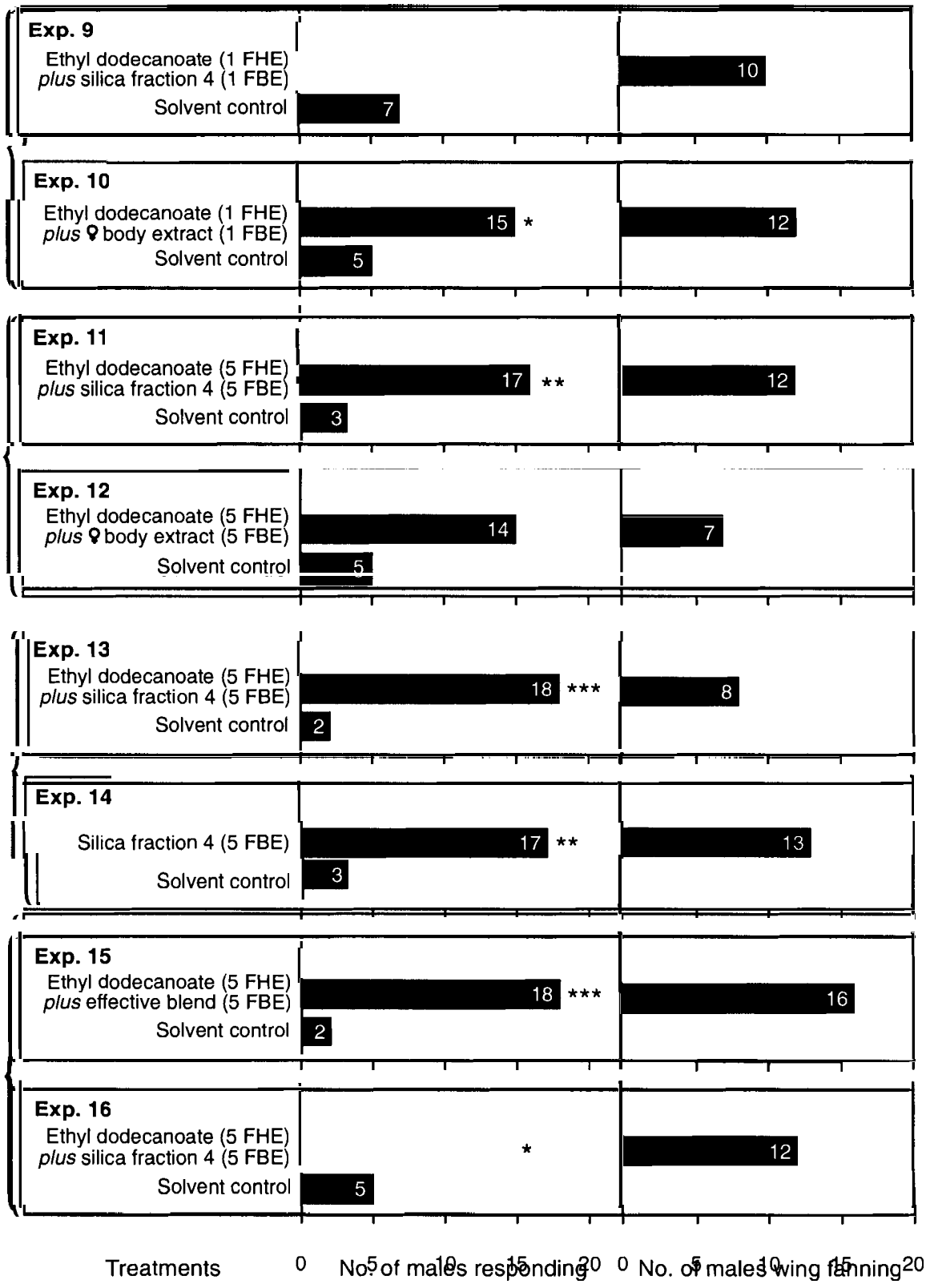
GC column	**Retention indices of:**			
	Component 1	**Component 2**	**Component 3**	**Component 4**
DB-5	2068	2089	2083	2108
DB-17	2314	2358	2381	2393
DB-210	2406	2429	2429	2481
DB-23	2583	2658	2700	2731
FFAP	2529	2608	2657	2657
Microanalytical treatments of body extract[1,2]	**Antennal response in GC-EAD recordings to:[3]**			
	Component 1	**Component 2**	**Component 3**	**Component 4**
Hydrogenation	absent	absent	absent	absent
Acetylation	present	present	present	present
Oxidation (PCC)	present	present	present, but smaller	present
Reduction ($NaBH_4$)	present	present	present	present
Reduction ($LiAlH_4$)	absent	absent	absent	absent
Deacetylation	present	present	absent	present

[1]Details of these standard treatments are described elsewhere (Huwyler, 1972; Corey and Suggs, 1975; Stanley, 1979; Bjostad et al., 1996; Millar and Haynes, 1998);

[2]Each treatment was repeated at least 2 times with different extracts;

[3]Each microtreated extract was tested with at least 3 male *G. flavicoxis* antennae in GC-EAD recordings.

Figure 2.4 Number of male *Glyptapanteles flavicoxis* that were attracted, or wing-fanned, in response to test stimuli in Y-tube olfactometer experiments 9-16. Abbreviations as in caption of figure 2.2; effective blend = combined HPLC fractions 16-20, 21-24, and 25-28 (see Figure 2.3). In each experiment, bars with asterisks (*) indicate a significant response to a particular treatment; heterogeneity χ^2 test with Yates' correction for continuity, treatment versus control; *P < 0.05; **P < 0.01; ***P < 0.001. Note: (1) Experiments grouped by brackets were run in parallel; (2) one male in experiment 12 did notrespond to test stimuli.


Exp. 9
Ethyl dodecanoate (1 FHE) plus silica fraction 4 (1 FBE)
Solvent control
7
10
Exp. 10
Ethyl dodecanoate (1 FHE) plus ♀ body extract (1 FBE)
Solvent control
15 *
5
12
Exp. 11
Ethyl dodecanoate (5 FHE) plus silica fraction 4 (5 FBE)
Solvent control
17 **
3
12
Exp. 12
Ethyl dodecanoate (5 FHE) plus ♀ body extract (5 FBE)
Solvent control
14
5
7
Exp. 13
Ethyl dodecanoate (5 FHE) plus silica fraction 4 (5 FBE)
Solvent control
18 ***
2
8
Exp. 14
Silica fraction 4 (5 FBE)
Solvent control
17 **
3
13
Exp. 15
Ethyl dodecanoate (5 FHE) plus effective blend (5 FBE)
Solvent control
18 ***
2
16
Exp. 16
Ethyl dodecanoate (5 FHE) plus silica fraction 4 (5 FBE)
Solvent control
*
5
12
Treatments
0 5 10 15 20
No. of males responding
0 5 10 15 20
No. of males wing fanning

Female body extract at 5 FBE plus ethyl dodecanoate was not attractive (Figure 2.4, Experiment 12), suggesting that this dose might have exceeded a biologically relevant threshold. In experiments 13 and 14, silica fraction 4 with or without ethyl dodecanoate appeared equally attractive to males.

In GC-EAD analyses of HPLC fractions of silica fraction 4, component 3 was present in fractions 16-20 (elution time: 4-5 min), components 2 and 4 (not separable) were present in fractions 21-24 (elution time: 5-6 min), and component 1 was present in fractions 25-28 (elution time: 6-7 min) (Figure 2.3). In Y-tube olfactometers, all fractions with one or more EAD-active components recombined at 5 FBE, together with ethyl dodecanoate, significantly attracted males (Figure 2.4, Experiment 15; Figure 2.6, Experiment 25). This effective blend was no longer attractive to males, when fractions 16-20 (containing component 3), 21-24 (containing components 2 and 4), or fractions 25-28 (containing component 1) were lacking (Figure 2.5; Experiments 17-22). Ethyl dodecanoate by itself, or in combination with HPLC fractions 16-20, failed to consistently attract males or to elicit wing-fanning responses (Figure 2.2, Experiment 7; Figure 2.5, Experiments 23, 24; Figure 2.6, Experiments 26, 27).

In experiment 28 (Figure 2.6), silica fraction 4 applied on a paper strip (15 x 1 cm) in a Y-tube's side arm prompted strong anemotactic and wing-fanning responses by males (see also Experiment 14), but failed to do so, with or without ethyl dodecanoate, when pipetted on a filter paper disc (4.3 cm diam.) at a side arm's orifice in parallel experiments 29 and 30. In contrast, 2 live females caged at a side arm's orifice were significantly attractive to males (Experiment 31).

Figure 2.5 Number of male *Glyptapanteles flavicoxis* that were attracted, or wing-fanned, in response to test stimuli in Y-tube olfactometer experiments 17-24. Abbreviations as in caption of figures 2.2 and 2.4. In each experiment, bars with asterisks (*) indicate a significant response to a particular treatment; heterogeneity χ^2 test with Yates' correction for continuity, treatment versus control; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Note: (1) Experiments grouped by brackets were run in parallel; (2) one male in experiment 17 did not respond to test stimuli.

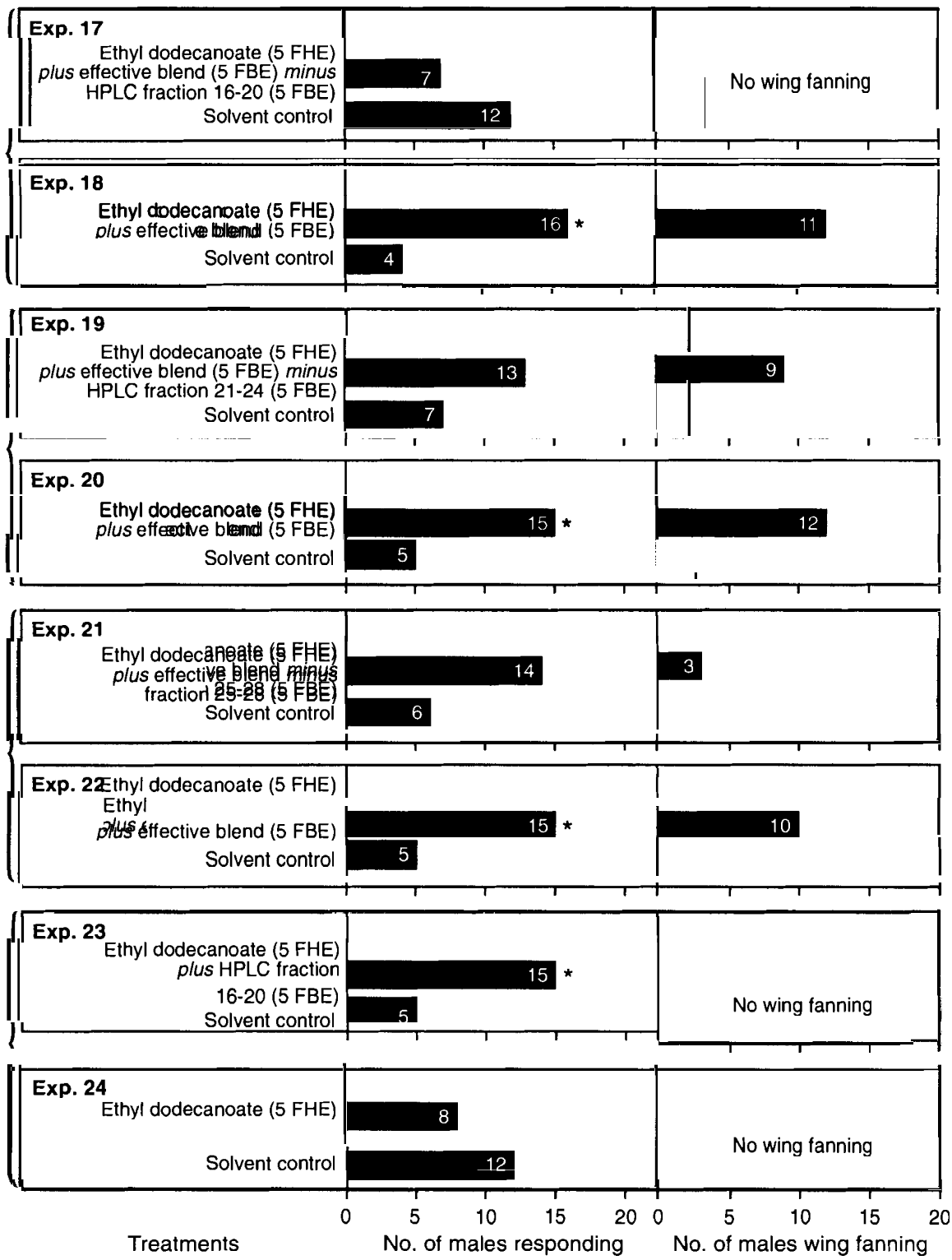
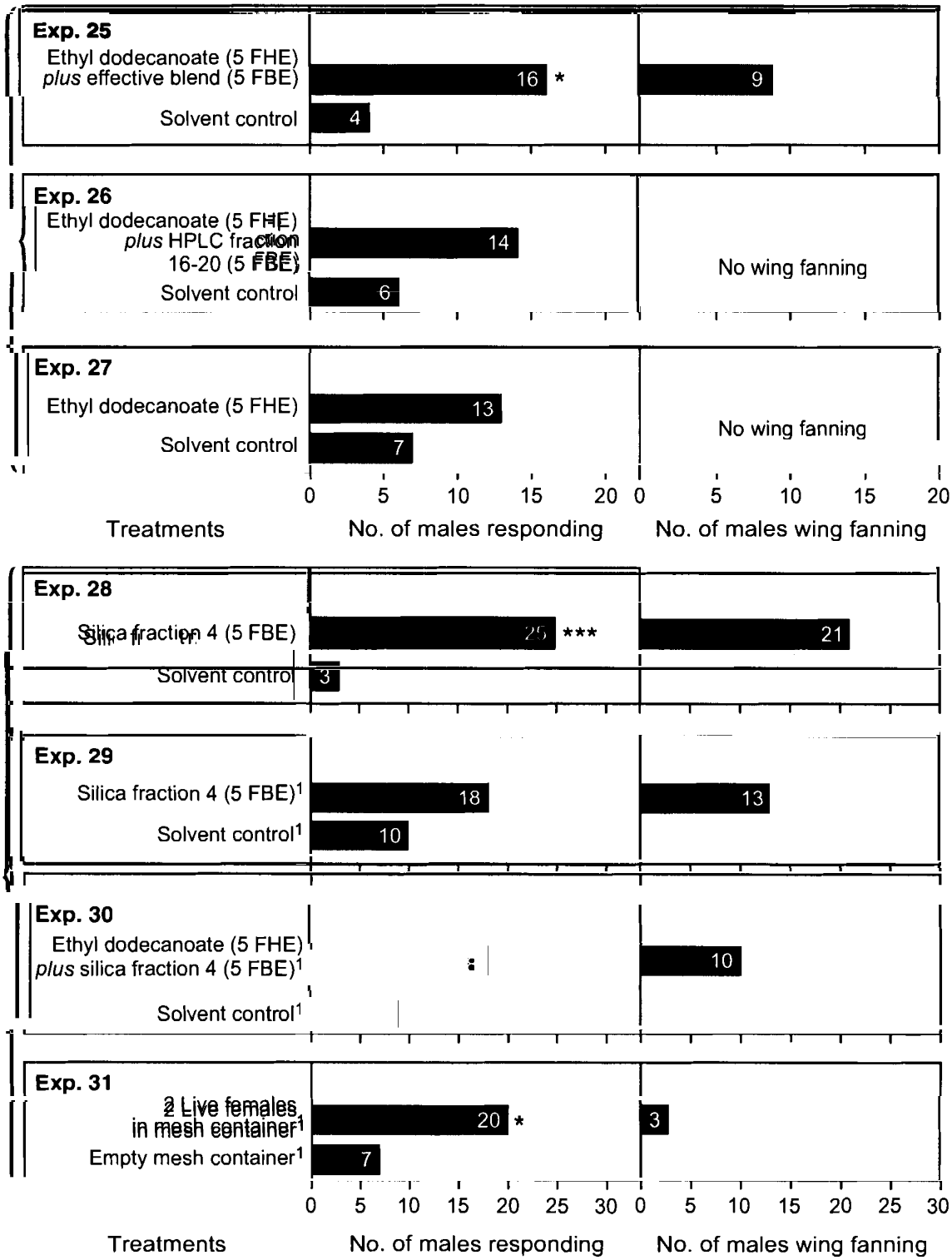

Exp. 17
Ethyl dodecanoate (5 FHE) plus effective blend (5 FBE) minus HPLC fraction 16-20 (5 FBE)
Solvent control
7
12
No wing fanning
Exp. 18
Ethyl dodecanoate (5 FHE) plus effective blend (5 FBE)
Solvent control
16 *
4
11
Exp. 19
Ethyl dodecanoate (5 FHE) plus effective blend (5 FBE) minus HPLC fraction 21-24 (5 FBE)
Solvent control
13
7
9
Exp. 20
Ethyl dodecanoate (5 FHE) plus effective blend (5 FBE)
Solvent control
15 *
5
12
Exp. 21
Ethyl dodecanoate (5 FHE) plus effective blend minus HPLC fraction 25-28 (5 FBE)
Solvent control
14
6
3
Exp. 22
Ethyl dodecanoate (5 FHE) plus effective blend (5 FBE)
Solvent control
15 *
5
10
Exp. 23
Ethyl dodecanoate (5 FHE) plus HPLC fraction 16-20 (5 FBE)
Solvent control
15 *
5
No wing fanning
Exp. 24
Ethyl dodecanoate (5 FHE)
Solvent control
8
12
No wing fanning
0 5 10 15 20
0 5 10 15 20
Treatments
No. of males responding
No. of males wing fanning

Figure 2.6 Number of male *Glyptapanteles flavicoxis* that were attracted, or wing-fanned, in response to test stimuli in Y-tube olfactometer experiments 25-31. Abbreviations as in caption of figures 2.2 and 2.4. In each experiment, bars with asterisks (*) indicate a significant response to a particular treatment; heterogeneity χ^2 test with Yates' correction for continuity, treatment versus control; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Note: (1) Experiments grouped by brackets were run in parallel; (2) one male in each of experiments 30 and 31 did not respond to test stimuli. [1]Test stimuli placed at the orifice of the Y-tube's side arms.


Exp. 25
Ethyl dodecanoate (5 FHE) plus effective blend (5 FBE)
16 *
9
Solvent control
4
Exp. 26
Ethyl dodecanoate (5 FHE) plus HPLC fraction 16-20 (5 FBE)
14
No wing fanning
Solvent control
6
Exp. 27
Ethyl dodecanoate (5 FHE)
13
No wing fanning
Solvent control
7
0 5 10 15 20 0 5 10 15 20
Treatments
No. of males responding
No. of males wing fanning
Exp. 28
Silica fraction 4 (5 FBE)
25 ***
21
Solvent control
3
Exp. 29
Silica fraction 4 (5 FBE)[1]
18
13
Solvent control[1]
10
Exp. 30
Ethyl dodecanoate (5 FHE) plus silica fraction 4 (5 FBE)[1]
10
Solvent control[1]
Exp. 31
2 Live females in mesh container[1]
20 *
3
Empty mesh container[1]
7
0 5 10 15 20 25 30 0 5 10 15 20 25 30
Treatments
No. of males responding
No. of males wing fanning

2.4 Discussion

Our data support the conclusion that female *G. flavicoxis* use a four-component pheromone blend that provokes strong close-range anemotactic attraction and wing-fanning responses by conspecific males (Figure 2.4, Experiment 14; Figure 2.6, Experiment 28). Response of males but not females to the pheromone (Figure 2.2, Experiment 4, 5) indicates that it is a sex pheromone rather than an aggregation pheromone. Failure of these four components to attract males over a distance of 10 cm (Figure 2.6, Experiment 29), coupled with attraction of males to live females over the same distance (Figure 2.6, Experiment 31), suggests that females use one or more additional pheromone components for long-range attraction of males. Similarly complex sexual communication has been reported for the parasitic wasp *Aphidius nigripes* (Aphididae) (McNeil and Brodeur, 1995; Marchand and McNeil, 2000). Body extracts of females provoked wing-fanning but not upwind flight by males, suggesting that female *A. nigripes* use both short- and long-range pheromone components.

Ethyl dodecanoate in the effluvia of female *G. flavicoxis* was a potential long-range pheromone component, but it did not affect the males' behavioural response in our experiments (Figure 2.4, Experiments 13, 14; Figure 2.6, Experiments 29, 30), and thus cannot be considered a pheromone component.

Video footage (graphical illustration not shown) revealed that females deposit, and males respond to pheromone on substrate. It is, however, not likely that females deposit a continuous trail, as bioassayed in experiment 1. Males of the braconid *As. reticulates* respond sporadically to substrate that females have frequented before,

suggesting that females deposit traces rather than trails of pheromone (Kamano et al., 1989). Similarly, in *G. flavicoxis*, substrate-borne pheromone may signal the presence of, rather than provide long-range directional cues toward, females (Figure 2.6; Experiments 29, 30).

Intriguingly, the close-range pheromone blend of *G. flavicoxis* is bifunctional, also eliciting wing-fanning responses by males. The males' strong wing-fanning response, however, was dependent upon their close distance to the pheromone source (e.g. Figure 2.4, Experiment 14; Figure 2.6, Experiment 28). Even caged live females (and their potential pheromone depositions on substrate) that remained inaccessible to males hardly elicited wing-fanning responses (Figure 2.6, Experiment 31). A strong wing-fanning response was also dependent upon the composition of the pheromone blend. It required the presence of component 3 and component(s) 1, or 2 and 4 (Figure 2.5).

Wing-fanning has been interpreted as a behaviour that facilitates the males' orientation toward females. As demonstrated with fine chalk dust in the ichneumonid *Campoletis sonorensis*, wing-fanning pulls air from front to rear, allowing directional orientation of males toward females (Vinson, 1972). This interpretation, however, does not explain completely why male *G. flavicoxis* were so discerning in their wing-fanning response to test stimuli (Figure 2.5). Males wing-fanned mostly in the presence of the complete pheromone blend, suggesting that they were motivated more by the quality of the female-produced signal, than prospects of improved anemotactic orientation toward females. If true, the males' wing-fanning could produce sound, possibly so specific that

the female could use it to recognize conspecific males and discern between prospective mates (Sivinski and Webb, 1989).

Identification of the close-range sex pheromone components was attempted but failed despite the large sample size (4,500 FE) that was analysed. Nonetheless, numerous micro-analytical treatments of, and electrophysiological recording with, pheromone extract (Table 2.1) suggested that all close-range sex pheromone components are unsaturated molecules of medium polarity, most likely esters. That these compounds remained below detection threshold of the mass spectrometer (~ 10 pg), even when 4,500 FE were analysed in a single injection, attests to the potency of the pheromone and the insects' sensitivity to it. Alternatively, the components are heat-labile, and defy identification by techniques involving gas chromatography.

3 SPECIES-SPECIFIC SEXUAL COMMUNICATION SYSTEMS PREVENT CROSS-ATTRACTION IN *G flavicoxis, G indiensis,* AND *G liparidis*

3.1 Introduction

Sexual communication in parasitoids is mediated mainly by pheromones that are emitted by females and induce searching, courtship and mating behavior by males (Quicke, 1997).

Specificity of the pheromone blend might serve as a reproductive isolating mechanism. Male sawfly parasitoids *Syndipnus gaspesianus* (Ichneumonidae) are not attracted to sympatric heterospecific female *S. rubiginosus* or their pheromone (Z)-9-hexadecenoate (Eller et al., 1984). Similarly, males of *Brachymeria intermedia* and *B. lasus* (Chalcididae) exhibit courtship behavior when exposed to pheromone extract of con- but not heterospecific females, suggesting that they use species-specific sex pheromones (Mohamed and Coppel, 1987a). Intriguingly, male *Melittobia digitata* (Eulophidae) emit sex pheromone that attracts conspecific females, but also cross-attracts female *M. femorata* and *M. australica*, suggesting that all three species use similar if not identical long-range pheromones. However, following antennal contact of prospective mates, heterospecifics are rejected, likely due to species-specific contact pheromones (Cônsoli et al., 2002).

Bioacoustic signals constitute alternative reproductive isolating mechanisms. Both *Diachasmimorpha longicaudata* and *D. kraussii* (Braconidae) use pheromonal, visual and

bioacoustic signals. Males are attracted to the female's cuticular pheromone, and respond with wing vibrational bioacoustic signals, which in turn increase the female's activity. The female's cuticular chemicals are similar across species, but acoustic signals of males appear to differ across the species (Rungrojwanich and Walter, 2000).

Congeners in the Lepidoptera often share pheromone components. Allopatric congeners may use the very same pheromone (Gries et al., 2002b), whereas sympatric congeners typically employ one or more additional pheromone components to maintain reproductive isolation (Gries et al., 1996). Similarly, the tortricid moths *Archips argyrospilus* and *A. mortuanus* share pheromone components in species-specific ratios (Cardé et al., 1977a).

Sexual communication in *G. flavicoxis* is mediated, in part, by a four-component close-range pheromone (Chapter 2). Pheromonal communications in *G. liparidis* and *G. indiensis* may be similarly complex but have not yet been investigated.

My objective was to determine whether *G. flavicoxis*, *G. indiensis*, and *G. liparidis* use species-specific components to confer specificity to their sexual communication systems.

3.2 Materials and methods

3.2.1 Experimental insects

Glyptapanteles flavicoxis and its host *L. dispar* were reared in the Global Forest Quarantine Facility at Simon Fraser University (SFU), as described in Chapter 2.2.1.

Cocoons of *G. indiensis* and *G. liparidis* were provided by the Beneficial Insects Introduction Research Laboratory (see above), and the Institute of Forest Entomology, Forest Pathology and Forest Protection, BOKU - University of Natural Resources and Applied Life Sciences, Vienna, Austria. Parasitoid cocoons with insects to be used in bioassays were transferred individually to capped plastic cups (30 ml) provisioned with sugar water-soaked cotton wicks. Rearing took place under a 16L:8D photoregime at 22-25 °C and 50-70% RH.

3.2.2 Acquisition of volatiles

Unmated 1- to 2-day-old female *G. flavicoxis*, *G. indiensis,* and *G. liparidis* were placed into vertical cylindrical Pyrex glass chambers (10 ID x 6 cm), and were provisioned with a sugar water-soaked cotton wick. Control chambers contained the same food source, but no parasitoids. A water aspirator drew humidified, charcoal-filtered air at a rate of 1.5-2 L/min for two days through the chamber and a glass column (14 x 1.3 cm OD) filled with 150 mg of Porapak Q (50-80 mesh, Waters Associates Inc., Milford, Massachusetts, USA). Volatiles were eluted from Porapak Q volatile traps with re-distilled pentane (2 ml). The extracts were concentrated under a stream of nitrogen such that 10 µl of extract contained one female hour equivalent (FHE) of volatile acquisition (= amount of volatiles released by 1 female during 1 hour).

3.2.3 Acquisition of pheromone extracts

Groups of 1- to 3-day-old female *G. flavicoxis*, *G. indiensis,* and *G. liparidis* were macerated in three separate vials that contained hexane (ca. 10 µl per female) placed on dry ice. Then the extracts were kept at room temperature for ~ 15 min. The supernatant was withdrawn, filtered through a small amount of glass wool in a pipette, and quantified to determine the volume representing one female body extract equivalent (FBE).

3.2.4 Y-tube olfactometer bioassays

Olfactometers and the general bioassay design are described in Chapter 2.2.5.

Experiments 1 and 2 tested behavioral responses by male *G. indiensis* and *G. liparidis* to effluvia and body extracts of conspecific females. Experiments 3-14 then tested body extract of female *G. flavicoxis*, *G. indiensis*, and *G. liparidis* for their potential cross-attractiveness to heterospecific males. Expecting consistent strong attraction of males to conspecific female pheromone, I tested the response of con- and heterospecific males in parallel experiments with alternating replicates. Thus, on any given bioassay day the males' lack of response to heterospecific female pheromone would likely be due to the non-attractiveness of the stimulus rather than the males' non-responsiveness, if males were responding strongly to conspecific female extracts.

The number of parasitoids responding to stimuli were analysed with the χ^2 goodness-of-fit test using Yates' correction for continuity ($\alpha = 0.05$), testing the null hypothesis that insects did not prefer treatment or control stimuli (Zar, 1996).

3.2.5 Analyses of *G. flavicoxis*, *G. indiensis*, and *G. liparidis* pheromone extracts

Aliquots of 1 FBE were analyzed by coupled gas chromatographic-electroantennographic detection (GC-EAD) (Arn et al., 1975; Gries et al., 2002a), employing a Hewlett Packard (HP) 5890A gas chromatograph equipped with a GC column (30 m x 0.25 or 0.32 mm ID) coated with DB-5, DB-17, DB-210, DB-23 or FFAP (J & W Scientific, Folsom, California 95630). For GC-EAD recordings, a male's head was severed and placed into the opening of a glass capillary electrode filled with saline solution (Staddon and Everton, 1980). One antenna with its tip removed by spring microscissors (Fine Science Tools Inc., North Vancouver, British Columbia Canada) was placed into the opening of a second (indifferent) electrode.

3.3 Results

In GC-EAD analyses of female *G. indiensis* pheromone extracts, male *G. indiensis* antennae responded to five components, one of which specific to *G. indiensis* (Gi-spec), and four shared with *G. flavicoxis* (Figure 3.1). Similarly, in GC-EAD analyses of female *G. liparidis* pheromone extract, male G. *liparidis* antennae responded to six components, two of which (Gl-specl and Gl-spec2) specific to *G. liparidis*, and four in common with *G. flavicoxis* (Figure 3.1).

In Y-tube olfactometer experiments, female *G. indiensis* body extract at 1 FBE in combination with effluvium (1 FHE) elicited significant attraction and wing-fanning responses by conspecific males (Figure 3.2, Experiment 1). Similarly, female *G. liparidis* body extract (1 FBE) plus effluvium (1 FHE) elicited significant attraction and wing-fanning responses by conspecific males (Figure 3.2, Experiment 2). In experiments 3-14 (Figure 3.3), which were designed to test potential pheromonal cross-attraction

Figure 3.1 Electroantennographic detector (EAD: conspecific male antenna) responses to aliquots of female *Glyptapanteles flavicoxis* body extract (*top*), female *G. indiensis* body extract (*middle*), and female *G. liparidis* body extract (*bottom*). Chromatography: Hewlett Packard 5890A equipped with a DB-23 coated GC column (30 m x 0.25 mm ID); linear flow velocity of carrier gas: 35 cm/sec; injector and FID detector temperature: 220° C; temperature program: 1 min at 100°C, 10°C/min to 220°C. Note: Corresponding flame ionization detector (FID) traces of the gas chromatograph are omitted because all antennal-stimulatory compounds occurred below FID detection threshold.

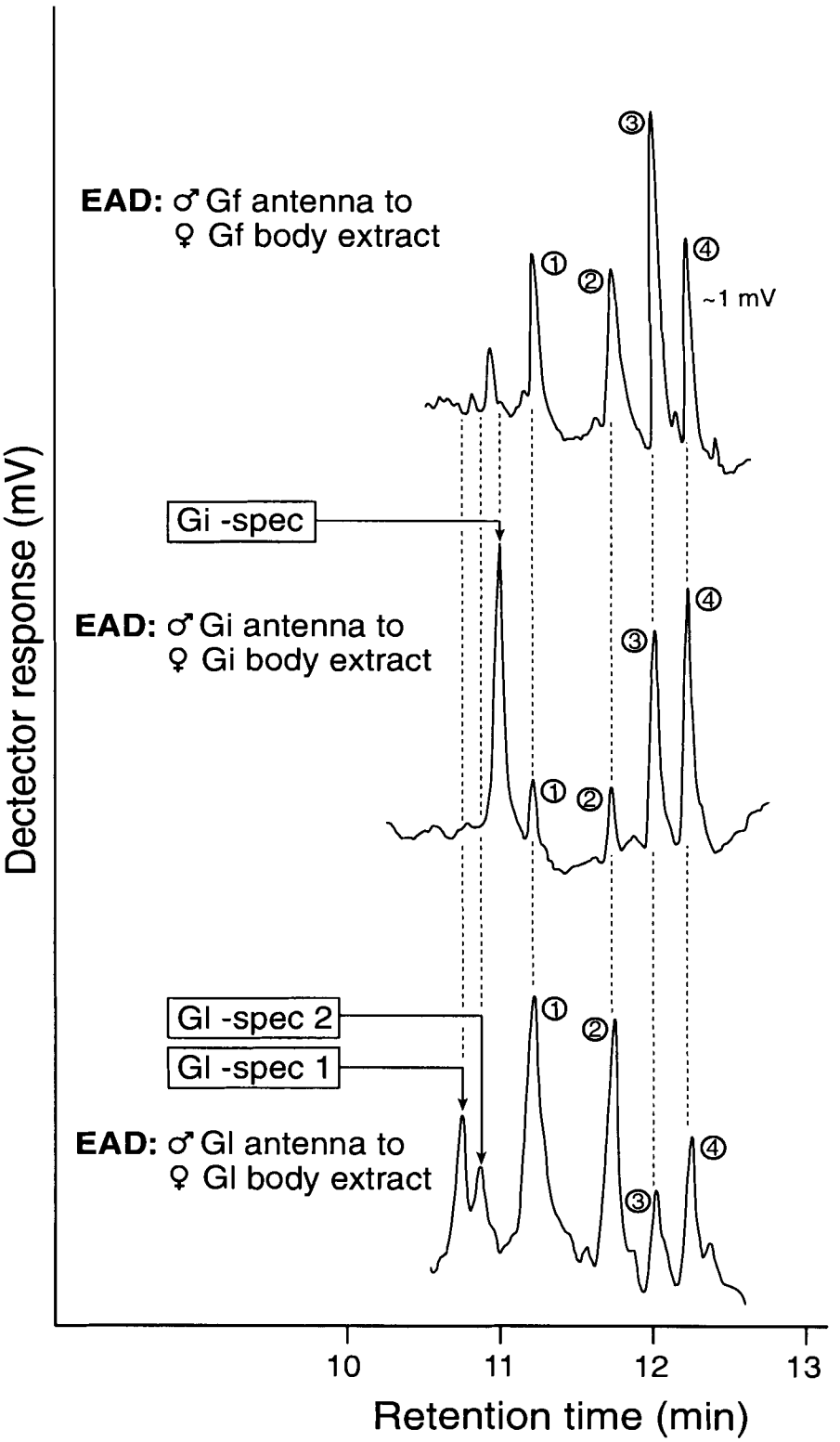

EAD: ♂ Gf antenna to
♀ Gf body extract
~1 mV
Gi -spec
EAD: ♂ Gi antenna to
♀ Gi body extract
Gl -spec 2
Gl -spec 1
EAD: ♂ Gl antenna to
♀ Gl body extract
Dectector response (mV)
10
11
12
13
Retention time (min)

Figure 3.2 Number of male *Glyptapanteles indiensis* and *G. liparidis* that were attracted, or wing-fanned, in response to test stimuli tested in Y-tube olfactometer experiments 1 and 2. 1 FHE = one female hour equivalent = pheromone component(s) released by one female during one hour; 1 FBE = one female body extract equivalent = pheromone component(s) contained in the extract of one macerated female body. In each experiment, bars with an asterisk (*) indicate a significant response to a particular treatment; χ^2 test with Yates' correction for continuity, treatment versus control; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

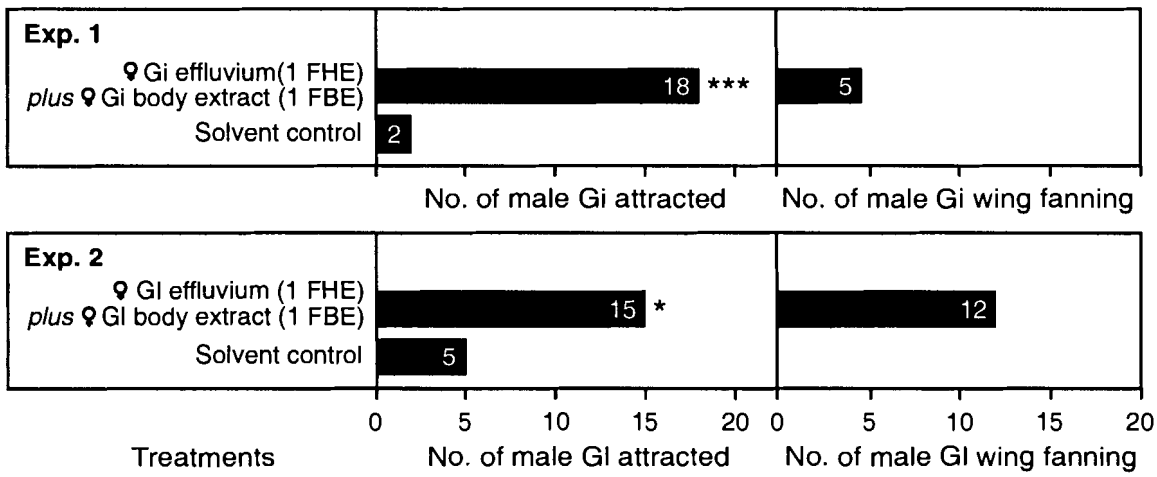
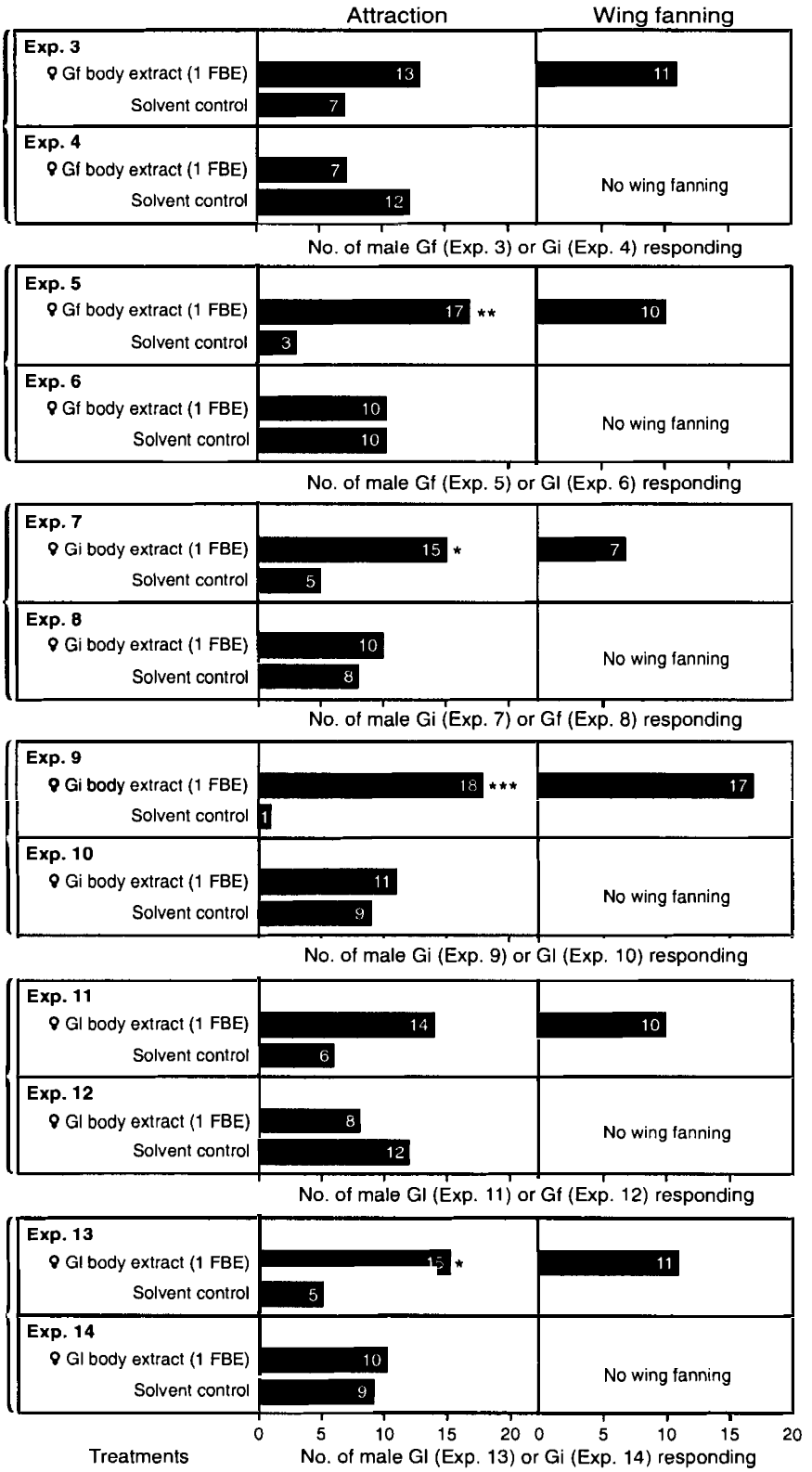

Exp. 1
♀ Gi effluvium(1 FHE)
plus ♀ Gi body extract (1 FBE)
Solvent control
18
***
5
2
No. of male Gi attracted
No. of male Gi wing fanning
Exp. 2
♀ GI effluvium (1 FHE)
plus ♀ GI body extract (1 FBE)
Solvent control
15
*
12
5
0
5
10
15
20
0
5
10
15
20
Treatments
No. of male GI attracted
No. of male GI wing fanning

Figure 3.3 Number of male *Glyptapanteles flavicoxis*, *G. indiensis*, and *G. liparidis* that were attracted, or wing-fanned, in response to test stimuli tested in Y-tube olfactometer experiments 3-14. 1 FBE = one female body extract equivalent = pheromone component(s) contained in the extract of one macerated female body. In each experiment, bars with an asterisk (*) indicate a significant response to a particular treatment; heterogeneity χ^2 test with Yates' correction for continuity, treatment versus control; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Note: (1) Experiments grouped by brackets were run in parallel; (2) one male in each of experiments 4, 9 and 14, and 2 males in experiment 8 did not respond to test stimuli.


Attraction
Wing fanning
Exp. 3
♀ Gf body extract (1 FBE)
13
11
Solvent control
7
Exp. 4
♀ Gf body extract (1 FBE)
7
No wing fanning
Solvent control
12
No. of male Gf (Exp. 3) or Gi (Exp. 4) responding
Exp. 5
♀ Gf body extract (1 FBE)
17 **
10
Solvent control
3
Exp. 6
♀ Gf body extract (1 FBE)
10
No wing fanning
Solvent control
10
No. of male Gf (Exp. 5) or Gl (Exp. 6) responding
Exp. 7
♀ Gi body extract (1 FBE)
15 *
7
Solvent control
5
Exp. 8
♀ Gi body extract (1 FBE)
10
No wing fanning
Solvent control
8
No. of male Gi (Exp. 7) or Gf (Exp. 8) responding
Exp. 9
♀ Gi body extract (1 FBE)
18 ***
17
Solvent control
1
Exp. 10
♀ Gi body extract (1 FBE)
11
No wing fanning
Solvent control
9
No. of male Gi (Exp. 9) or Gl (Exp. 10) responding
Exp. 11
♀ Gl body extract (1 FBE)
14
10
Solvent control
6
Exp. 12
♀ Gl body extract (1 FBE)
8
No wing fanning
Solvent control
12
No. of male Gl (Exp. 11) or Gf (Exp. 12) responding
Exp. 13
♀ Gl body extract (1 FBE)
15 *
11
Solvent control
5
Exp. 14
♀ Gl body extract (1 FBE)
10
No wing fanning
Solvent control
9
0 5 10 15 20 0 5 10 15 20
Treatments
No. of male Gl (Exp. 13) or Gi (Exp. 14) responding

among species, body extract of female *G. flavicoxis* elicited attraction and/or wing-fanning responses by conspecific males (Experiments 3, 5), but not by heterospecific male *G. indiensis* (Experiment 4) or *G. liparidis* (Experiment 6). Furthermore, body extract of female *G. indiensis* elicited attraction and wing-fanning responses by conspecific males (Experiments 7, 9), but not by heterospecific male *G. flavicoxis* (Experiment 8) or *G. liparidis* (Experiment 10). Finally, body extract of female *G. liparidis* elicited attraction and/or wing-fanning responses by conspecific males (Experiments 11, 13), but not by heterospecific male *G. flavicoxis* (Experiment 12) or *G. indiensis* (Experiment 14).

3.4 Discussion

My data support the hypothesis that *G. indiensis*, *G. liparidis*, and *G. flavicoxis* share (candidate) pheromone components but use additional components to confer specificity to their sexual communication. The same four pheromone components that are present in body extracts of female *G. flavicoxis*, and elicit antennal and behavioural responses from conspecific males (Figure 3.1; Danci et al., 2006), are also present in body extracts of female *G. indiensis* and *G. liparidis* (Figure 3.1). However, whether all of them are pheromone components in *G. indiensis* and *G. liparidis*, as in *G. flavicoxis*, is yet to be determined.

The presence of the same four components in all three species is indicative of phylogenetic relatedness, and supports taxonomic placement of the three species as congeners. Comparable volatile or pheromone blends of sympatric *G. flavicoxis* and *G. indiensis* were expected, but the very similar volatile blend of allopatric *G. liparidis* is surprising. It is suggestive of a common ancestor that has given rise to all three species.

Analogously, *Elatophilus hebraicus* (Hemiptera: Anthocoridae), a hemipteran predator of *Matsucoccus* scales (Homoptera: Matsucoccidae), not only respond to the pheromone of the sympatric prey species *Matsucoccus josephi*, but also to the pheromone of two allopatric *Matsucoccus* prey species, suggesting the kairomonal response of *E. hebraicus* has evolved during sympatric speciation of the genus *Matsucoccus* (Dunkelblum et al., 1996).The complete lack of pheromonal cross-attraction among the three *Glyptapanteles* species (Figure 3.3) is likely due to the species-specific components in *G. indiensis* (Gi-spec) and *G. liparidis* (Gl-spec1 and/or Gl-spec2). Should these compounds be part of the respective pheromone blends, they would be synomones that enhance attraction of conspecifics while simultaneously inhibiting the response of heterospecifics. Synomonal activity of pheromone components has been well documented in the Coleoptera and Lepidoptera. The heliothine moths *Heliothis zea*, *H. virescens* and *H. subflexa* (Lepidoptera: Noctuidae) share (*Z*)-11-hexadecenal as a common pheromone component, whereas (*Z*)-9-hexadecenal in *H. zea*, (*Z*)-9-hexadecenal and (*Z*)-11-hexadecen-1-ol in *H. subflexa*, and (*Z*)-9-tetradecenal in *H. virescens* enhance attractiveness and species-specificity of the respective pheromone blends (Vetter and Baker, 1983, 1984; Vickers, 2002). Similarly, bark beetle aggregation pheromones contain components that interrupt the pheromonal response of competing species. Sympatric *Ips paraconfusus* and *Ips pini* (Coleoptera: Scolytidae), for example, infest the same host, but components of their respective pheromones inhibit cross-attraction (Birch and Haynes, 1982).

It is also conceivable that Gi-spec in *G. indiensis*, and Gl-spec1 and Gl-spec2 in *G. liparidis*, are non-pheromonal constituents in their respective communication systems, serving the single role of reducing cross-attraction of heterospecifics. Such a concept has

been proposed for nun moth, *Lymantria monacha* (Lepidoptera: Lymantriidae), and its sympatric congener *L. dispar*, both using (+)-disparlure as a pheromone component. Attraction of male *L. dispar* to (+)-disparlure is inhibited in the presence of (–)-disparlure (Klimetzek et al., 1976; Cardé et al., 1977b; Plimmer et al., 1977), which is likely produced as a non-pheromonal constituent by female *L. monacha* to enhance the specificity of sexual communication (Hansen, 1984).

To assign non-pheromonal or synomonal roles to Gi-spec in *G. indiensis*, and to Gl-specl or Gl-spec2 in *G. liparidis*, will require their isolation and bioassay testing. Although they occur well below GC or GC-mass spectrometric detection thresholds, they can be separated from other candidate pheromone components by high performance liquid chromatography (HPLC), carefully monitoring HPLC fractions by GC-EAD (see Danci et al., 2006; Chapter 2).

Reproductive isolating mechanisms in insects operate at multiple levels (Birtch and Haynes, 1982). At the behavioural and physiological level, species-specific sexual communication systems contribute to prezygotic reproductive isolation of *G. flavicoxis*, *G. indiensis,* and *G. liparidis*, irrespective of their allopatric or sympatric occurrence.

4 CONCLUSIONS

Hymenopteran parasitoids employ short and/or long-range sex pheromones, which attract potential mates and play a role during courtship behaviour. In my thesis, I have investigated pheromonal comunication in three braconid congeners: *G. flavicoxis*, *G. indiensis* and *G. liparidis*.

Based on my data, the following conclusions can be drawn:

1. Female *G. flavicoxis*, *G. indiensis,* and *G. liparidis* deposit pheromone by pressing sporadically their abdominal tip on the substrate. These "deposits," or body extracts of females, provoke substrate-antennation, wing-fanning and short-range anemotactic attraction responses by conspecific males.
2. Female *G. flavicoxis*, *G. indiensis,* and *G. liparidis* all deploy species-specific short-range sex pheromones that attract con- but not heterospecific males.
3. The short-range sex pheromone of female *G. flavicoxis* comprises four components, which are all necessary to elicit short-range attraction and wing-fanning responses by conspecific males.
4. Pheromone extracts of *G. indiensis* and *G. liparidis* contained the four *G. flavicoxis* pheromone components, but also contained additional components that likely contributed to species-specific blends.
5. Wing-fanning as the most conspicuous element of male courtship behaviour occurs only in the presence of live females, pheromonal deposits from live females, or body extracts of females. Wing-fanning by males might produce sound that provides females with species and mate recognition cues.

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