INTRASPECIFIC COMMUNICATION OF THE COMMON BED BUG *Cimex lectularius* LINNAEUS (HEMIPTERA: CIMICIDAE)

by

Eric Daniel Siljander

B.Sc. (Biology: Ecology and Evolution), Simon Fraser University, 2004

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF PEST MANAGEMENT

In the Department of Biological Sciences

© Eric Daniel Siljander 2007

SIMON FRASER UNIVERSITY

2007

All rights reserved. This work may not be reproduced in whole or in part, by photocopy or other means, without permission of the author.
APPROVAL

Name: Eric Daniel Siljander

Degree: Master of Pest Management

Title of Thesis:

Intraspecific communication of the common bed bug *Cimex lectularius* Linnaeus (Hemiptera: Cimicidae)

Examining Committee:

Chair: Dr. E. Elle, Associate Professor

Dr. G. Gries, Professor, Senior Supervisor
Department of Biological Sciences, S.F.U.

Dr. C. Lowenberger, Associate Professor
Department of Biological Sciences, S.F.U.

Dr. D. Huber, Assistant Professor
Ecosystem Science and Management Program,
University of Northern British Columbia
Public Examiner

7 August 2007
Date Approved
DECLARATION OF PARTIAL COPYRIGHT LICENCE

The author, whose copyright is declared on the title page of this work, has granted to Simon Fraser University the right to lend this thesis, project or extended essay to users of the Simon Fraser University Library, and to make partial or single copies only for such users or in response to a request from the library of any other university, or other educational institution, on its own behalf or for one of its users.

The author has further granted permission to Simon Fraser University to keep or make a digital copy for use in its circulating collection (currently available to the public at the “Institutional Repository” link of the SFU Library website <www.lib.sfu.ca> at: <http://ir.lib.sfu.ca/handle/1892/112>) and, without changing the content, to translate the thesis/project or extended essays, if technically possible, to any medium or format for the purpose of preservation of the digital work.

The author has further agreed that permission for multiple copying of this work for scholarly purposes may be granted by either the author or the Dean of Graduate Studies.

It is understood that copying or publication of this work for financial gain shall not be allowed without the author’s written permission.

Permission for public performance, or limited permission for private scholarly use, of any multimedia materials forming part of this work, may have been granted by the author. This information may be found on the separately catalogued multimedia material and in the signed Partial Copyright Licence.

The original Partial Copyright Licence attesting to these terms, and signed by this author, may be found in the original bound copy of this work, retained in the Simon Fraser University Archive.

Simon Fraser University Library
Burnaby, BC, Canada

Revised: Spring 2007
ABSTRACT

Intraspecific pheromonal communication was investigated in the common bed bug, *Cimex lectularius* Linnaeus (Hemiptera: Cimicidae). In dual choice laboratory olfactometer experiments, *C. lectularius* showed specific preference for paper discs that had been exposed to conspecifics of the same developmental stage. These male- and juvenile-produced contact pheromones may have the contrasting functions of marking refugia for development and growth (juveniles) or mate-encounter (adults), but result in the same phenomenon, the aggregation of conspecifics.

Using dual choice experiments, gas chromatography-mass spectrometry and silica gel fractionation, 14 candidate pheromone components were identified in headspace volatiles from the *C. lectularius* laboratory colony. A synthetic blend of octanal, nonanal, decanal, (E)-2-hexenal, (E)-2-octenal, (E,E)-2,4-octadienal, (E,Z)-2,4-octadienal, benzaldehyde, benzyl alcohol, benzyl acetate, (+)-limonene, (−)-limonene, sulcatone and geranylacetone elicited significant behavioural responses from juveniles, adult males and virgin adult females. Synthetic aggregation pheromone may be useful in detecting *C. lectularius* in human dwellings.

**Keywords:** *Cimex lectularius*, Hemiptera, Cimicidae, bed bug, blood-feeding, ectoparasite, communication, semiochemical, pheromone, aggregation, arrestment, attractant.
ACKNOWLEDGEMENTS

I would like to thank my senior supervisor, Dr. Gerhard Gries, for giving me the opportunity to research such a fascinating insect. I am especially grateful for his thorough guidance and support throughout this research and during the preparation of my thesis.

I thank Mrs. Regine Gries, for patiently helping me with everything that my research entailed, from where to find a screwdriver, to how to perform complex chemical analyses. All of her help was invaluable.

I thank my committee member, Dr. Carl Lowenberger, for all his constructive criticism, and for his thoroughly helpful review of this thesis. I also wish to thank Dr. Dezene Huber for serving as public examiner at my thesis defense.

I am indebted to Dr. Harold Harlan for his expert advice on bed bug rearing and handling techniques, and for his “endless” supply of *C. lectularius* that helped make this research possible.

I thank Dr. Grigori Khaskin for his assistance with chemistry and for synthesizing *(E,E)*- and *(E,Z)*-2,4-octadienal for Chapter 3 of my thesis.

This research could not have progressed unless the bed bugs were fed. For that, I am most grateful to Cory Campbell, Tom Cowan, Christine De Pape, Zaid Jumean, Charlene Wood and Nathan Woodbury for helping me feed the bedbugs.

I thank Dan Penman for being an excellent research assistant and for his help running experiments for Chapter 2 of my thesis.
I would also like to thank Dr. Eberhard Kiehlmann, Zaid Jumean, and two anonymous reviewers for constructive comments on manuscripts that have arisen from Chapter 2 and Chapter 3 of my thesis.

I thank all my colleagues over the years for brainstorming sessions, helpful input, support and camaraderie that has helped me get through this research.

I thank my family for their unwavering support of my academic pursuits, no matter how long they take. My special thanks go to Lindsay Williams for her patience, support, and for being my reason to do anything.

I thank the organizations that made my research possible by supplying funding. This study was supported, in part, by the Thelma Finlayson and Simon Fraser University Master of Pest Management Graduate Entrance Scholarships, a Graduate Fellowship from Simon Fraser University, a Finlandia Club Scholarship, and by a Natural Sciences and Engineering Research Council of Canada – Industrial Research Chair to Dr. Gerhard Gries with SC Johnson Canada, Pherotech International Inc. and Global Forest Science (GF-18-2007-226; GF-18-2007-227) as the industrial sponsors.
# TABLE OF CONTENTS

- Approval ........................................................................................................... ii
- Abstract ............................................................................................................... iii
- Acknowledgements ............................................................................................ iv
- Table of Contents ............................................................................................... vi
- List of Figures ...................................................................................................... viii
- List of Tables ....................................................................................................... xi

## Chapter 1  Intraspecific communication in haematophagous Hemiptera ........1
  1.1 Intraspecific Communication ........................................................................1
  1.1.1 Behaviour of the Triatominae .................................................................1
  1.1.2 Sex Pheromones in Triatominae .............................................................2
  1.1.3 Aggregation Pheromones in Triatominae ..............................................2
  1.1.4 Alarm Pheromones in Triatominae .........................................................3
  1.2 Semiochemical Parsimony and Multifunctional Pheromones ....................3
  1.3 Biology and Ecology of Bed Bugs ...............................................................4
    1.3.1 Classification and Distribution ...............................................................4
    1.3.2 Life History of *Cimex lectularius* .......................................................5
    1.3.3 Mating Behaviour ..................................................................................8
    1.3.4 Feeding Behaviour ................................................................................8
    1.3.5 Harbourage Use and Aggregation Behaviour ......................................9
    1.3.6 Dispersal Behaviour .............................................................................10
  1.4 Pest Status of *Cimex lectularius* ...............................................................10
    1.4.1 Effect on Hosts ......................................................................................10
    1.4.2 Potential as a Vector of Human Pathogens .........................................11
    1.4.3 Recent Resurgence ...............................................................................11
  1.5 Current Knowledge about Intraspecific Communication in *C. lectularius* ....12
  1.6 Research Objectives .................................................................................14
  1.7 Reference List .............................................................................................15

## Chapter 2  Evidence for male- and juvenile-specific contact pheromones ....21
  2.1 Abstract ........................................................................................................21
  2.2 Introduction ...................................................................................................21
  2.3 Materials and Methods ..............................................................................23
    2.3.1 Origin and Maintenance of *Cimex lectularius* Colony ......................23
    2.3.2 Acquisition of Contact Pheromones ...................................................23
    2.3.3 Still-Air Olfactometer Experiments ...................................................24
Chapter 3  Identification of an airborne aggregation pheromone blend ..........38
  3.1 Abstract .................................................................................38
  3.2 Introduction ...........................................................................38
  3.3 Materials and Methods ............................................................39
      3.3.1 Experimental Insects .........................................................39
      3.3.2 Acquisition of Volatiles .....................................................40
      3.3.3 Analytical Methods .........................................................40
      3.3.4 Olfactometer Experiments .................................................41
      3.3.5 Statistical Analyses ..........................................................46
  3.4 Results .................................................................................46
  3.5 Discussion ............................................................................62
  3.6 Reference List ........................................................................67

Chapter 4  Concluding summary ......................................................70
  4.1 Conclusions ............................................................................70
  4.2 Implications for Pest Management ............................................71
LIST OF FIGURES

Figure 1.1 Life cycle of the common bed bug, *Cimex lectularius* Linnaeus (Hemiptera: Cimicidae). Modified from illustration by Scott Charlesworth with support from Catherine Hill and John MacDonald. .................................................................6

Figure 2.1 Response of juvenile, adult male, and adult female *Cimex lectularius* in still-air olfactometers to paper discs previously exposed for six days to 10 conspecific juveniles (experiments 1-3), 10 mated adult males (experiment 4-6) or 10 mated adult females (experiments 7-9). Number of insects responding to each stimulus is given within bars; number of insects not responding in each experiment is given in parentheses. For each experiment, *P*-values are provided in the figure; \( \chi^2 \) test with Yates correction for continuity; \( \alpha = 0.05 \) ..................26

Figure 2.2 Response of juvenile and adult female *Cimex lectularius* in still-air olfactometers to inaccessible paper discs previously exposed for six days to 10 conspecific juveniles (experiment 10) or 10 adult males (experiment 11). Number of insects responding to each stimulus is given within bars; number of insects not responding in each experiment is given in parentheses. For each experiment, *P*-values are provided in the figure; \( \chi^2 \) test with Yates correction for continuity; \( \alpha = 0.05 \) .................................................................28

Figure 2.3 Response of juvenile *Cimex lectularius* in still-air olfactometers to aliquots of 1440 contact pheromone hour equivalents (CPHE) (experiment 12) and 480 CPHE (experiment 13) of methanol extract of paper discs previously exposed to juvenile conspecifics. 1 CPHE = contact pheromone deposited by one *C. lectularius* during 1 hr. Number of insects responding to each stimulus is given within bars; number of insects not responding in each experiment is given in parentheses. For each experiment, *P*-values are provided in the figure; \( \chi^2 \) test with Yates correction for continuity; \( \alpha = 0.05 \). The same volume of methanol (200 µl, experiment 12; 95 µl, experiment 13) was applied to treatment and control stimuli. ........................................30

Figure 3.1 Response of adult male (experiment 1), adult female (experiment 2) and juvenile (experiments 3, 4) *Cimex lectularius* in still-air olfactometers to aliquots [100 or 2000 bug hour equivalents (BHE)] of Porapak Q colony jar aeration extracts. 1 BHE = volatiles released by one *C. lectularius* during 1 hr. Number of insects responding to each stimulus is given within bars; number of insects
not responding in each experiment is given in parentheses. For each experiment, $P$-values are provided in the figure; $\chi^2$ test with Yates correction for continuity; $\alpha = 0.05$. The same volume of pentane (7.5 $\mu$l) was applied to treatment and control stimuli.

Figure 3.2 Response of juvenile *Cimex lectularius* in still-air olfactometers to aliquots (200 BHE; see Figure 3.1) of Porapak Q colony jar aeration extracts (experiment 5), or to silica gel fractions (200 BHE) of the same extract (experiments 6-11). Number of insects responding to each stimulus is given within bars; number of insects not responding in each experiment is given in parentheses. For each experiment, $P$-values are provided in the figure; $\chi^2$ test with Yates correction for continuity; $\alpha = 0.05$. Experiments 6-11 were run in parallel; the same volume of pentane (10 $\mu$l, experiment 5; 50 $\mu$l, experiments 6-11) was applied to treatment and control stimuli.

Figure 3.3 Response of juvenile (experiment 12), adult male (experiment 13), mated adult female (experiment 14) and virgin adult female (experiment 15) *Cimex lectularius* in still-air olfactometers to a synthetic blend (SB) of 14 candidate pheromone components at 200 BHE. Number of insects responding to each stimulus is given within bars; number of insects not responding in each experiment is given in parentheses. For each experiment, $P$-values are provided in the figure; $\chi^2$ test with Yates correction for continuity; $\alpha = 0.05$. The SB consisted of three saturated aldehydes [octanal, nonanal, decanal], four unsaturated aldehydes [(E)-2-hexenal, (E)-2-octenal, (E,E)-2,4-octadienal, (E,Z)-2,4-octadienal], one aromatic aldehyde [benzaldehyde], two monoterpenes [(+)-limonene, (−)-limonene], two ketones [sulcatone, geranylacetone], one acetate [benzyl acetate] and one aromatic alcohol [benzyl alcohol]. The same volume of pentane (10-15 $\mu$l) was applied to treatment and control stimuli.

Figure 3.4 Response of juvenile *Cimex lectularius* in still-air olfactometer experiments 16-22 to synthetic blends (SB) at 200 BHE, lacking groups of candidate pheromone components. Number of insects responding to each stimulus is given within bars; number of insects not responding in each experiment is given in parentheses. For each experiment, $P$-values are provided in the figure; $\chi^2$ test with Yates correction for continuity; $\alpha = 0.05$. All experiments were run in parallel; the same volume of pentane (15 $\mu$l) was applied to treatment and control stimuli.

Figure 3.5 Response of juvenile *Cimex lectularius* in still-air olfactometer experiments 23-36 to synthetic blends (SB) at 200 BHE, lacking single candidate pheromone components. Number of insects responding to each stimulus is given within bars; number of insects not responding in each experiment is given in parentheses. For each experiment, $P$-values are provided in the figure; $\chi^2$ test with Yates correction for continuity; $\alpha = 0.05$. All experiments were run in
parallel; the same volume of pentane (15 μl) was applied to treatment and control stimuli.............................56

Figure 3.6  Response of juvenile *Cimex lectularius* in still-air olfactometer experiments 37-44 to new synthetic blends (NSB; blend as described in caption of Figure 3.3 without octanal) at 200 BHE, lacking one or more candidate pheromone components. Number of insects responding to each stimulus is given within bars; number of insects not responding in each experiment is given in parentheses. For each experiment, *P*-values are provided in the figure; χ² test with Yates correction for continuity; *α* = 0.05. All experiments were run in parallel; the same volume of pentane (10 μl) was applied to treatment and control stimuli.............................58

Figure 3.7  Mean (+ SE) proportion of 10 juvenile *Cimex lectularius* per replicate (10 replicates) responding in cage olfactometer experiments 45 and 46 to a synthetic blend (SB; blend composition as described in caption of Figure 3.3) at 2000 BHE (experiment 45) or SB in combination with *C. lectularius* juvenile contact pheromone at 720 contact pheromone hour equivalents (CPHE) (experiment 46). 1 CPHE = contact pheromone deposited by one *C. lectularius* during 1 hr. For each experiment, *P*-values are provided in the figure; Wilcoxon paired-sample test; *α* = 0.05. The same volume of pentane (50 μl) for SB application or methanol (100 μl) for CPHE application was applied to treatment and control stimuli............60
LIST OF TABLES

Table 3.1 Summary of experimental insects and stimuli tested in laboratory still-air and cage bioassays. .................................................................42
CHAPTER 1
INTRASPECIFIC COMMUNICATION IN
HAEMATOPHAGOUS HEMIPTERA

1.1 Intraspecific Communication

Intraspecific communication is based on signal exchange between members of the same species. Intraspecific signals may be of several modalities: chemical; bioacoustic; visual; etc. Chemicals which mediate interactions between organisms are broadly referred to as semiochemicals (= message-bearing chemicals) (Nordlund and Lewis 1976). Semiochemicals used for intraspecific communication are classified as pheromones (Blum 1996). The modern definition of pheromone is “a substance, or substances, that is/are secreted by an animal or plant to the outside that cause a specific reaction in a receiving individual of the same species” (Nordlund and Lewis 1976, Borden 1985). Here I will discuss intraspecific communication of haematophagous species of the Hemiptera that are adapted to humans as hosts.

1.1.1 Behaviour of the Triatominae

The sub-family Triatominae (Hemiptera: Reduviidae) contains species that are haematophagous on vertebrate hosts, including humans. Some are vectors of the flagellate parasite *Trypanosoma cruzi*, the cause of Chagas’ disease (Schofield and Patterson 1977; Lorenzo Figuieras *et al.* 1994; Cruz López and Morgan 1995; Lorenzo and Lazzari 1996; Lorenzo Figuieras and Lazzari 1998; Cruz López *et al.* 2001). Species that can reside in human dwellings, such as *Triatoma infestans*, become active at night to
forage for blood meals from human hosts, and spend days assembled in shaded and
tightly confined refugia (Schofield 1979; Lorenzo Figuieras et al. 1994). These habits are
typical of haemtophagous hemipterans that are adapted to human hosts.

1.1.2 Sex Pheromones in Triatominae

Putative sex pheromones in Triatominae have been described, but evidence is
equivocal (Cruz López et al. 2001). In the species Rhodnius prolixus and T. infestans,
there is evidence that males are attracted to conspecific mating pairs. It is unclear,
however, which gender is actually emitting attractive compounds (Baldwin et al. 1971;
Schofield and Moreman 1976; Manrique and Lazzari 1995; De Brito Sánchez et al. 1995;
Cruz López et al. 2001).

1.1.3 Aggregation Pheromones in Triatominae

Many different aggregation factors have been identified in the species T.
infestans, R. prolixus and T. mazzottii (Cruz López et al. 2001). Feces of T. infestans
have been found to contain an assembly pheromone that attracts and arrests nymphs
(Schofield and Patterson 1977; Lorenzo Figuieras et al. 1994). Feces of T. mazzottii seem
to contain two pheromones: one from male feces that attracts other males and one from
female and juvenile feces that attracts unfed nymphs and females (Ondarza et al. 1986).
However, synthetic blends of four components identified from feces of both T. infestans
and T. mazzottii showed no consistent attractiveness (Cruz López and Morgan 1995).
Nitrogenous compounds in bug feces, such as ammonia and ammonium chloride,
reportedly attract T. infestans (Taneja and Guerin 1997; Fontan et al. 2000; Cruz López et
al. 2001). Compounds in feces of T. infestans and T. mazzottii that elicited behavioural
responses were extracted with polar solvents; however resulting extracts were only weakly attractive (Cruz López and Morgan 1995). Lorenzo and Lazzari (1996) suggested that fecal pheromone of T. infestans may serve as a chemical marker to aid in refuge selection. However, ultimate reasons for aggregation behaviour in the Triatominae have yet to be investigated (Cruz López et al. 2001).

1.1.4 Alarm Pheromones in Triatominae

When irritated, triatomine bugs release isobutyric acid from the Brindley’s glands (Cruz López et al. 2001). Isobutyric acid is believed to function as a defensive compound, because topical application caused paralysis in R. prolixus nymphs (Kalin and Barrett 1975; Schofield 1979). Rhodnius prolixus adults exposed to isobutyric acid vapour became aroused and disrupted aggregations (Kalin and Barrett 1975). Although these results led to the suggestion that isobutyric acid is an alarm pheromone, other experiments have shown that low concentrations were actually attractive to R. prolixus (Schofield 1979). The intraspecific function of isobutyric acid is not clear, but the results reported suggest that it may act as a concentration dependent multifunctional pheromone.

1.2 Semiochemical Parsimony and Multifunctional Pheromones

Most hemipterans have prominent scent glands that produce diverse chemicals which often serve as defensive compounds (Staddon 1979; Aldrich 1988; Millar 2005). Arthropods have demonstrated an ability to exploit their semiochemicals with “considerable parsimonious versatility” (Blum 1996). Due to constraints on biosynthesis of semiochemicals, there is strong selection pressure to evolve the use of single components for multiple functions (Borden 1985; Blum 1996). Multifunctional
pheromones have been reported in Hemiptera. In the pentatomid *Eurydema rugosa*, (E)-2-hexenal has been shown to be attractive at low concentrations and repellent at high concentrations (Ishiwatari 1974, 1976). In another pentatomid, *Nezara viridula*, n-tridecane was attractive at low concentrations and repellent at high concentrations (Lockwood and Story, 1985). Therefore, any investigations attempting to identify an insect’s pheromones mediating a specific behaviour should not disregard those already identified that mediate other behaviours, as they potentially may serve multiple functions.

1.3 Biology and Ecology of Bed Bugs

1.3.1 Classification and Distribution

Cimicidae are a family of the true bugs (Hemiptera) that include the human bed bugs, bat bugs, chicken bugs, swallow bugs and pigeon bugs. All genera are ectoparasitic on warm-blooded vertebrates and all life stages are haematophages (Usinger 1966). Bed bugs are 5-8 mm in length, dorsoventrally flattened, lack wings, and have a reddish-brown or mahogany-like colouration (Borror *et al.* 1989). The abdomen is broadly oval; however, males have a slightly tapered posterior where the reproductive organs are housed. Juveniles resemble adults but lack fully developed reproductive organs and are less sclerotized, giving them a lighter straw-like colouration (Usinger 1966). While feeding, the abdomen expands to accommodate the blood meal by stretching intersegmental membranes and ventral hunger folds (adults) or the membranous base and ventral side (juveniles) (Usinger 1966).

There are three species of bed bugs: the common bed bug, *Cimex lectularius* L.; the tropical bed bug, *Cimex hemipterus* F., and *Leptocimex boueti* (Usinger 1966). Of
these, *L. boueti* has the most restricted distribution, being found only in Western Africa (Usinger 1966). *Cimex hemipterus* is tropicopolitan and rarely extends into northern or southern temperate regions (Usinger 1966). *Cimex lectularius* is cosmopolitan, but is primarily found throughout the northern temperate region with patchy distribution in the tropics and southern temperate regions (Usinger 1966). Although there have been reports of *C. hemipterus* in Florida (Hixson 1943), *C. lectularius* is the only bed bug commonly found in North America.

1.3.2 Life History of *Cimex lectularius*

As do all true bugs, *Cimex lectularius* undergoes hemimetabolous development that includes an egg stage, five nymphal instars each needing a blood meal to moult, and an adult stage (Johnson 1941; Usinger 1966) (Figure 1.1). The time to complete a life cycle is dependent upon the frequency of blood meals available and the environmental temperature, which directly affects metabolism and development rate (Johnson 1941; Usinger 1966). Under typical room temperatures (18-23°C) and with blood meals *ad libitum*, total development time from oviposition to oviposition can range from 58 to 128 days (Johnson 1941; Omori 1941). However, if a host is not readily available, *C. lectularius* can survive between 3-8 months between feedings (Johnson 1941; Omori 1941). During fasting periods, growth, development, and reproduction cease. Adults live for 3-6 months and females lay their small (~ 1 mm), oblong, whitish eggs individually at a rate of 2-5 eggs per day with up to 200-500 in their lifetime (Johnson 1941). All stages of *C. lectularius* can survive through a wide range of temperatures and humidities. The thermal death point is 45°C (Omori 1941) and the developmental- hatching and
Figure 1.1 Life cycle of the common bed bug, *Cimex lectularius* Linnaeus (Hemiptera: Cimicidae). Modified from illustration by Scott Charlesworth with support from Catherine Hill and John MacDonald.
activity threshold is 13-15°C (Kemper 1936; Johnson 1941; Usinger 1966).

1.3.3 Mating Behaviour

All Cimicidae mate extragenitally by traumatic insemination (Carayon 1966). The male mounts the female, pierces her abdomen with a needle-like intromittent organ (paramere) and inseminates into the female’s body cavity. Female *C. lectularius*, like other cimicid females, have adapted a specialized paragenital system (Carayon 1966; Stutt and Siva-Jothy 2001; Morrow and Arnqvist 2003; Reinhardt *et al.* 2003). It includes specific structures that receive the male’s intromittent organ (ectospermalege), receive sperm (mesospermalege), store sperm which have migrated through the hemocoel (seminal conceptacles) and that conduct spermatozoa to the ovaries (spermodes) (Carayon 1966).

*Cimex lectularius* is polygamous and can mate throughout its lifespan. Individuals in laboratory colonies have high re-mating rates and most females are fertilized (Johnson 1941; Stutt and Siva-Jothy 2001). However, female specimens collected from natural populations are often unfertilized, suggesting that mating in nature is less frequent (Johnson 1941).

1.3.4 Feeding Behaviour

*Cimex lectularius* is nocturnal and individuals leave their harbourage to forage for a blood meal when their host is asleep (Johnson 1941; Usinger 1966). It is unclear how *C. lectularius* locates a host from a distance. Studies have investigated the attractiveness of host-associated cues such as heat, humidity, blood, carbon dioxide (CO₂), muscle and subcutaneous tissue, liver, bile, skin, hair, perspiration, sebum and cerumen (Hase 1917;
Rivnay 1930, 1932; Sioli 1937; Marx 1955; Aboul-Nasr and Erakey 1967, 1968a). Only one stimulus – heat – was consistently found to be attractive and able to induce the biting reflex. *Cimex lectularius* is attracted to heat above ambient temperature and can detect a difference of 1-2°C (Rivnay 1932; Sioli 1937; Aboul-Nasr and Erakey 1967). However, heat is only effective in attracting from a range of a few centimetres (Rivnay 1932; Usinger 1966). Sebum and cerumen were also found to attract *C. lectularius*, but this only occurred at a very short range (Rivnay 1932). Many investigators believe that *C. lectularius* cannot detect a host beyond a few centimetres (Rivnay 1932; Kemper 1936; Usinger 1966), but one study claimed that they could detect a human from a distance of 150 cm (Marx 1955). Marx (1955) attributed this to heat and CO₂, which she also reported to be attractive.

Once *C. lectularius* finds a host, it uses its piercing-sucking mouthparts to probe through the skin and into the tissue to search for a suitable blood vessel (Dickerson and Lavoipierre 1959). Once such a vessel is located, the maxillae enter the lumen of the vessel and blood flows up the food canal (Dickerson and Lavoipierre 1959). Feeding to repletion takes 3-15 minutes (Girault 1905), and when complete insects leave the host and return to their harbourage (Usinger 1966).

### 1.3.5 Harbourage Use and Aggregation Behaviour

During the day, *C. lectularius* hide in refugia such as bedding, mattresses, bed frames, furniture, cracks and crevices in the wall, under wallpaper, baseboards, window frames, picture frames, electrical outlets, switch covers and other tightly confined areas (Usinger 1966; Borror *et al.* 1989). They prefer still-air microhabitats that are dry, offer rough substrates and at least partial darkness (Hase 1917). They use negative phototaxis.
and positive thigmotaxis as guiding mechanisms to lead them to tight, well-protected crevices (Usinger 1966; Aboul-Nasr and Erakey, 1968b). Often *C. lectularius* forms aggregations within refugia that consist of many individuals of mixed stage and gender (Kemper 1936; Johnson 1941; Usinger 1966). Refugia where these aggregations occur are termed “brood centers” or harbourages (Kemper 1936; Usinger 1966). *Cimex lectularius* will return to these harbourages after each blood meal and may remain there for up to several weeks while digestion, defecation, moulting, mating and oviposition take place (Kemper 1936; Johnson 1941; Usinger 1966). Harbourages accumulate fecal matter, egg shells and exuviae (Usinger 1966). It remains unclear how *C. lectularius* find aggregates of conspecífics or relocate their harbourages.

### 1.3.6 Dispersal Behaviour

Dispersal behaviour is one of the most poorly understood aspects of *C. lectularius* ecology (Reinhardt and Siva-Jothy 2007). Active dispersal of *C. lectularius*, such as walking between rooms, is restricted to the structure in which they reside (Mellanby 1938). Passive dispersal via humans is most important in transporting *C. lectularius* long distances (Reinhardt and Siva-Jothy 2007). They can be moved on clothing, luggage, and furniture, and may travel long distances on buses, trains, ships and airplanes (Usinger 1966; Reinhardt and Siva-Jothy 2007).

### 1.4 Pest Status of *Cimex lectularius*

#### 1.4.1 Effect on Hosts

*Cimex lectularius* is considered a pest of annoyance, causing allergic reactions to saliva injected while biting (Hecht 1930; Borror *et al.* 1989). Reactions are usually minor
(Usinger 1966; Ryckman 1985), and approximately 20% of people have no reaction (Kemper 1936). However, common reactions can include pruritis, edema, erythema, papular urticaria and bullous eruptions (Ryckman 1985; Tharakaram 1999; Leverkus et al. 2006). Anaphylaxis is rare but has been reported (Parsons 1955). Bite wounds may also be prone to secondary infection (Reinhardt and Siva-Jothy 2007).

1.4.2 Potential as a Vector of Human Pathogens

Due to the nature of blood feeding in *C. lectularius*, there is concern about their potential ability to transmit human pathogens (Usinger 1966). Many human pathogens naturally occur in *C. lectularius* (Burton 1963), but there is no evidence of their transmission. For example, the hepatitis B virus (HBV) persists in the body and feces of *C. lectularius* for up to six weeks, although it is unknown if any remaining virus is infectious (Blow et al. 2001; Silverman et al. 2001). In transmission experiments, Jupp et al. (1991) found that biological multiplication and biological transmission of HBV does not occur in *C. lectularius*. However, *C. lectularius* may mechanically transmit viruses by regurgitating ingested blood during interrupted feedings, feces being deposited on wounds or mucosal surfaces, or bugs being crushed on wounds or mucosal surfaces (Jupp et al. 1991; Blow et al. 2001; Silverman et al. 2001).

1.4.3 Recent Resurgence

*Cimex lectularius* was a common human pest until World War II, when the widespread availability of persistent insecticides, such as DDT, led to a significant decrease of infestations in developed countries (Cornwell 1974; Busvine 1980; Boase 2001; Doggett et al. 2004; Hwang et al. 2005). Since the late 1990’s, regulatory agencies
and pest management companies in Canada, USA, Great Britain, Western Europe and Australia have recorded increases in *C. lectularius* interceptions and infestations (Krueger 2000; Boase 2001; Doggett et al. 2004; Hwang et al. 2005).

There are four main hypotheses for the recent resurgence of *C. lectularius* (summarized from Doggett et al. 2004):

i. Increased international travel – It provides *C. lectularius* with opportunities for passive dispersal;

ii. Changes in pest management practices - Pesticides and associated methods previously effective against *C. lectularius* are no longer available for use in most developed countries. Modern pesticides and strict regulation make effective control more difficult;

iii. Development of pesticide resistance - Ineffective treatments are leading to rapid development of resistance to registered pesticides; and

iv. Lack of knowledge – Unfamiliarity with *C. lectularius* has allowed it to “slip under the radar”, and a paucity of research has not resulted in new management tools.

1.5 **Current Knowledge about Intraspecific Communication in *C. lectularius***

The area of intraspecific communication in *C. lectularius* has received little attention. Initial investigations into the existence of pheromones were directed at the metathoracic scent glands and their secretions (Kemper 1936; Usinger 1966). The main constituents in scent glands of adult and juvenile *C. lectularius* are the unsaturated
aldehydes \((E)-2\)-hexenal and \((E)-2\)-octenal, along with the minor constituents acetaldehyde, 2-butanone and two unknown components (Collins 1968; Levinson et al. 1974a). Levinson and Bar Ilan (1971) bioassayed \((E)-2\)-hexenal and \((E)-2\)-octenal singly and in combination, and found that \textit{C. lectularius} did not aggregate in response to these chemicals. Also, when these components were tested in the same proportion as found in scent glands, aggregated insects quickly dispersed. Therefore, \((E)-2\)-hexenal and \((E)-2\)-octenal have been classified as the \textit{C. lectularius} alarm pheromone (Levinson and Bar Ilan 1971; Levinson et al. 1974a,b).

Marx (1955) tested the hypothesis that \textit{C. lectularius} is attracted to the odour of conspecifics by bioassaying paper discs previously exposed to bugs and unexposed discs. She found that the responders tended to aggregate under the previously exposed ("scented") paper discs more often than the control discs. Levinson and Bar Ilan (1971) repeated Marx’s experiments and confirmed her results. The so-called ‘assembling scent’ was extractible with methanol, yielding a relatively effective scent solution (Levinson and Bar Ilan 1971). No follow-up investigations have been reported, leaving the source, mode of action, and chemicals mediating the aggregation response unknown.
1.6 Research Objectives

My research objectives were:

1. To test the hypothesis that juvenile *C. lectularius* produce, and respond to, an aggregation pheromone;

2. To determine whether the pheromone of *C. lectularius* adults and juveniles (if shown to exist) is perceived by olfaction or contact chemoreception;

3. To determine if the pheromone of *C. lectularius* juveniles (if shown to exist) is extractible with methanol;

4. To investigate whether *C. lectularius* use an airborne pheromone; and if so

5. To identify the pheromone components.
1.7 Reference List


CHAPTER 2
EVIDENCE FOR MALE- AND JUVENILE-SPECIFIC CONTACT PHEROMONES

2.1 Abstract

Males and females of the common bed bug, *Cimex lectularius* Linnaeus (Hemiptera: Cimicidae), have been shown to produce, and respond to, an aggregation pheromone. I tested whether juvenile *C. lectularius* also produce and respond to aggregation pheromone, and whether the pheromone is perceived by contact chemoreception. In dual choice laboratory experiments, juveniles, but not adult males or females, preferred juvenile-exposed paper discs over control discs. Unlike juveniles, adult males and females preferred male-exposed paper discs over control discs. Neither juveniles, adult males nor adult females preferred female-exposed discs over control discs. When test stimuli were inaccessible, *C. lectularius* showed no preference. Adult male- and juvenile-specific contact pheromones may have the contrasting functions of marking shelters as safe refugia for development and growth (juveniles) or mate-encounter (adults), but result in the same phenomenon, the aggregation of conspecifics.

2.2 Introduction

The common bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae), has been an ectoparasitic pest of humans throughout recorded history (Usinger 1966;  

---

1 A modified version of this chapter has been accepted for publication in Entomologia Experimentalis et Applicata: Siljander, E., Penman, D., Harlan, H., and Gries, G. Evidence for male- and juvenile-specific contact pheromones of the common bed bug, *Cimex lectularius*. 

21
Panagiotakopulu and Buckland 1999; Reinhardt and Siva-Jothy 2007). Recently, *C. lectularius* has experienced a global resurgence (Krueger 2000; Boase 2001) with large economic impact due to costly control efforts, lost business and litigation (Doggett et al. 2004). Considering that all stages of *C. lectularius* bite and blood-feed, the need has arisen to investigate their communication ecology (Reinhardt and Siva-Jothy 2007). Synthetic pheromones may be exploited for detection of infestations within human dwellings.

According to previous reports (Marx 1955; Levinson and Bar Ilan 1971) adult male and female *C. lectularius* aggregate under paper discs previously exposed to adult conspecifics, suggesting the existence of adult pheromonal communication. Interestingly, Levinson and Bar Ilan (1971) stated – but showed no data – that fifth-instar juveniles only showed a slight response to such exposed discs. The so-called ‘assembling scent’ might have been an airborne or contact pheromone, and was extractible with methanol. Many other insects that inhabit enclosed microhabitats, such as cockroaches (Sakuma & Fukami 1993), firebrats (Tremblay and Gries 2003), silverfish (Woodbury and Gries 2007) and kissing bugs (Lorenzo Figueiras and Lazzari 1998) utilize substrate-bound contact pheromones for communication. These contact pheromones might be beneficial in microhabitats with little or no air movement, where airborne pheromone could cause habituation of the olfactory system.

Currently it is unknown, and thus I tested, whether (i) juvenile *C. lectularius* also produce and respond to aggregation pheromones; (ii) the pheromone of adults and of juveniles (if shown to exist) is perceived by olfaction or contact chemoreception; and (iii) juvenile-produced pheromone is also extractible with methanol.
2.3 **Materials and Methods**

2.3.1 **Origin and Maintenance of *Cimex lectularius* Colony**

*Cimex lectularius* were supplied by, or collected from, three sources: (1) Harold Harlan, Crownsville, MD, USA; (2) George Keeney, Ohio State University, Columbus, OH, USA; and (3) natural populations in human dwellings, Vancouver, BC, Canada.

Insects were kept in wide-mouth glass jars (9 × 9.5 cm) containing strips of cardboard folded accordion-style to provide a surface on which they could walk and oviposit.

Insects were reared at 21-28 °C, 25-50 % R.H., and a 16L:8D photoperiod. They were blood-fed through an artificial membrane (Montes *et al.* 2002) or on human volunteers.

The membrane system used heparinated chicken blood as a food source. For feeding on humans, groups of insects were placed in jars (3.5 × 5.5 cm) topped with nylon mesh and allowed to feed to repletion.

2.3.2 **Acquisition of Contact Pheromones**

Paper towel discs (9 cm diam.; folded eight times for corrugation) were exposed in Petri dishes (9 × 5 cm) for six days to 10 juvenile, 10 mated adult male, or 10 mated adult female *C. lectularius* at room temperature under a 16L:8D photoperiod. Such discs received up to 1440 contact pheromone hour equivalents (1 CPHE = contact pheromone deposited by one *C. lectularius* during 1 hr). To extract the contact pheromone, exposed paper discs were submerged in methanol (~3 ml; HPLC-grade) for ~30 min at room temperature. The supernatant was withdrawn with a pipette and stored at room temperature.
2.3.3 Still-Air Olfactometer Experiments

Responses of *C. lectularius* to test stimuli were bioassayed in still-air olfactometers because *C. lectularius* inhabit enclosed microhabitats with little or no air movement and avoid moving air (Kemper 1936; Johnson 1941; Usinger 1966; Siljander, personal observation). Each olfactometer consisted of three glass chambers (each 3.5 \times 10 \text{ cm ID}) linearly interconnected by glass tubes (each 2.5 \times 1 \text{ cm ID}) (Takács and Gries 2001). Test stimuli were randomly assigned to one of the two lateral chambers, each containing a paper towel disc (9 cm diam.; folded eight times for corrugation) with a tab (2.5 \times 0.7 \text{ cm}) extending through to the central chamber to facilitate inter-chamber movement of bioassay insects. For each of 20-35 replicates in each of the following 13 experiments, one naive *C. lectularius* was released in the central chamber of the olfactometer 3 hr before the end of scotophase, when *C. lectularius* are most active (Mellanby 1939). An insect was classed as a responder when it was found 16-18 hr later resting on a paper disc or tab. Non-responders were not included in statistical analyses. All experiments were conducted at 22-24 °C, 25-40 % R.H., under an 18-hr photoperiod of 3D:15L.

Experiments 1-9 tested the response of juveniles [4\textsuperscript{th}-5\textsuperscript{th} instar], adult males or adult females to paper previously exposed to juveniles (experiments 1-3), mated adult males (experiments 4-6) or mated adult females (experiments 7-9). Experiments 10-11 tested whether the response required physical contact with the stimulus by suspending the insect-exposed and control paper discs from the ceiling of the lateral chambers, making them inaccessible to responding *C. lectularius*. Experiments 12-13 tested aliquots (100-200 \text{ µl}) of methanol extracts of juvenile-exposed paper containing up to 1440 CPHE
(experiment 12) and up to 480 CPHE (experiment 13) to determine whether such aliquots had a behavioural effect comparable to that of exposed paper. Aliquots of extract were pipetted onto the discs, with treatment and control discs receiving the same volume of solvent.

2.3.4 Statistical Analyses

Numbers of *C. lectularius* responding to treatment and control stimuli in all experiments were analyzed with the $\chi^2$ goodness-of-fit test, using Yates correction for continuity ($\alpha = 0.05$) (Zar 1999).

2.4 Results

In olfactometer experiments 1-9, *C. lectularius* showed specific preference for paper discs that had been exposed to conspecifics of the same developmental stage. Juveniles, but not adult males or adult females, preferred juvenile-exposed paper discs over unexposed control discs (Figure 2.1, experiments 1-3). Unlike juveniles, adult males and females preferred adult male-exposed paper discs over control discs (Figure 2.1, experiments 4-6). Neither juveniles, adult males nor adult females preferred adult female-exposed paper discs over control discs (Figure 2.1, experiments 7-9). When test stimuli were inaccessible, *C. lectularius* failed to show a preference for either treatment or control discs (Figure 2.2). Juveniles preferred methanol extracts of paper discs exposed to juveniles over extracts of unexposed paper discs when the extracts were tested at aliquots of 480 CPHE, but not when tested at 1440 CPHE (Figure 2.3).
Figure 2.1  Response of juvenile, adult male, and adult female *Cimex lectularius* in still-air olfactometers to paper discs previously exposed for six days to 10 conspecific juveniles (experiments 1-3), 10 mated adult males (experiment 4-6) or 10 mated adult females (experiments 7-9). Number of insects responding to each stimulus is given within bars; number of insects not responding in each experiment is given in parentheses. For each experiment, $P$-values are provided in the figure; $\chi^2$ test with Yates correction for continuity; $\alpha = 0.05$. 
Juveniles

Exp. 1

Adult ♀♂

Exp. 2

Adult ♀♀

Exp. 3

Juvenile - exposed paper

18 (0)

P < 0.001

Unexposed paper

2

Exp. 4

Male - exposed paper

10 (1)

Exp. 5

Male - unexposed paper

9

P = 0.004

Exp. 6

Female - exposed paper

9 (6)

Exp. 7

Female - unexposed paper

9

P = 0.817

Exp. 8

Test stimuli  Number of C. lectularius responding

0 10 20 0 10 20 0 10 20 0 10 20
Figure 2.2 Response of juvenile and adult female *Cimex lectularius* in still-air olfactometers to inaccessible paper discs previously exposed for six days to 10 conspecific juveniles (experiment 10) or 10 adult males (experiment 11). Number of insects responding to each stimulus is given within bars; number of insects not responding in each experiment is given in parentheses. For each experiment, *P*-values are provided in the figure; $\chi^2$ test with Yates correction for continuity; $\alpha = 0.05$. 
Exp. 10
Juvenile-exposed paper (inaccessible) 9 (1)
Unexposed paper (inaccessible) 12

Exp. 11
Male-exposed paper (inaccessible) 8 (2)
Unexposed paper (inaccessible) 11

Test stimuli

Number of *C. lectularius* juveniles (Exp. 10) or adult females (Exp. 11) responding

$P = 0.663$

$P = 0.646$
Figure 2.3 Response of juvenile *Cimex lectularius* in still-air olfactometers to aliquots of 1440 contact pheromone hour equivalents (CPHE) (experiment 12) and 480 CPHE (experiment 13) of methanol extract of paper discs previously exposed to juvenile conspecifics. 1 CPHE = contact pheromone deposited by one *C. lectularius* during 1 hr. Number of insects responding to each stimulus is given within bars; number of insects not responding in each experiment is given in parentheses. For each experiment, *P*-values are provided in the figure; $\chi^2$ test with Yates correction for continuity; $\alpha = 0.05$. The same volume of methanol (200 µl, experiment 12; 95 µl, experiment 13) was applied to treatment and control stimuli.
Exp. 12
Juvenile contact pheromone in methanol (1440 CPHE)
Methanol control

Test stimulus
Number of juvenile C. lectularius responding

Exp. 13
Juvenile contact pheromone in methanol (480 CPHE)
Methanol control

Test stimulus
Number of juvenile C. lectularius responding

$P = 0.118$

$P = 0.004$
2.5 Discussion

My data support the conclusion that aggregations of juvenile and adult *C. lectularius* (Kemper 1936; Usinger 1966) are formed, in part, in response to adult- and juvenile-specific contact aggregation pheromones. My results confirm previous observations that fifth-instar nymphs showed a weak response to paper previously exposed to adults (Levinson and Bar Ilan 1971). In other synanthropic gregarious insects, such as cockroaches, firebrats and silverfish, both adults and juveniles produce and respond to the same signal (Ishii and Kuwahara 1967, 1968; Tremblay and Gries 2003; Woodbury and Gries 2007). Juvenile-specific signalling is rare, but has been reported in both hemimetabolus and holometabolus insects. Pheromone emitted by nymphs of the desert locust, *Schistocerca gregaria*, attracts conspecific nymphs but not adults (Obeng-Ofori et al. 1993; Torto et al. 1996). Larvae of the eastern tent caterpillar, *Malacosoma americanum*, deposit a trail pheromone along silk trails that directs tent mates to food finds (Fitzgerald 1976). Cocoon-spinning larvae of the codling moth, *Cydia pomonella*, produce an aggregation pheromone that attracts pupation site-seeking larvae (Duthie et al. 2003; Jumean et al. 2004, 2005a,b, 2007) and differs from the sex pheromone produced by adult females (Roelofs et al. 1971; Witzgall et al. 2001).

The function of stage-specific insect pheromones is poorly understood. In holometabolous Lepidoptera, they might facilitate the contrasting needs of resource-exploiting or pupation site-seeking larvae and of adults engaging in sexual communication. In hemimetabolous *S. gregaria*, they might help maintain age-group cohesion and reduce resource competition between stages (Uvarov 1966; Obeng-Ofori et al. 1993; Hassanali et al. 2005). Neither phenomenon applies to *C. lectularius*. The fact
that male-produced aggregation pheromone arrests both adult females and males implies that it may have evolved as a sex pheromone which was secondarily exploited by mate-seeking males, and thus became an aggregation pheromone. If so, adult male- and juvenile-specific contact pheromones would have the contrasting functions of marking shelters as safe refugia for development and growth (juveniles) or mate-encounter (adults), but result in the same phenomenon, the aggregation of conspecifics.

The lack of response to adult female-exposed paper by adult males or adult females (Figure 2.1, experiments 8-9) differs from previous findings (Marx 1955; Levinson and Bar Ilan 1971), but may be attributed to the fact that test stimuli were produced using mated, rather than virgin, females. Levinson and Bar Ilan (1971) showed that both mated and virgin \textit{C. lectularius} responded to female-exposed paper discs, but did not reveal the mating status of females used to produce the test stimulus. Mated females cease to respond to airborne pheromones, as shown in a separate study (Chapter 3). Thus, the mating status of females may also affect their deposition of contact pheromone.

Levinson and Bar Ilan (1971) were able to extract adult-specific contact pheromone with methanol. My methanol extracts of juvenile-specific contact pheromone also elicited behavioural responses, but were not as effective as pheromone deposited by juveniles on paper discs. Similar results occurred in attempts to identify the fecal aggregation pheromone of \textit{T. infestans} and \textit{T. mazzottii}. Methanol-extracted feces had reduced attractiveness, but resulting extracts were only weakly attractive (Cruz López and Morgan 1995). The methanol solubility of both adult- and juvenile-specific contact pheromones in \textit{C. lectularius} suggests molecules of similar polarity.
The adult male- and juvenile-specific contact pheromones (Figure 2.2) likely serve as harbourage markers, arresting *C. lectularius* in harbourages that provide shelter from harsh environmental conditions, protection from natural enemies and access to potential mates (Wertheim *et al.* 2005). Harbourage marking behaviour has also been reported in other synanthropic insects, such as cockroaches (Denzer *et al.* 1988; Stejskal 1997), firebrats (Tremblay and Gries 2003), silverfish (Woodbury and Gries 2007) and kissing bugs (Lorenzo and Lazzari 1996; Lorenzo Figueiras and Lazzari 1998).

If contact pheromones accumulate on surfaces through prolonged exposure to *C. lectularius*, surfaces repeatedly walked on by *C. lectularius* also may become marked. *Cimex lectularius* that “commute” between their harbourage and a sleeping host may deposit contact pheromone intentionally or passively as a trail. A trail between harbourage and host would reduce foraging time, thereby reducing the risk of detection and predation. It has been hypothesized that *C. lectularius* use a ‘trail pheromone’ (Kemper 1936; Johnson 1941), but no evidence has been produced to date.

Future studies should identify the contact pheromones produced by adult male and juvenile *C. lectularius* and investigate whether these pheromones are used for marking foraging trails.
2.6 Reference List


CHAPTER 3
IDENTIFICATION OF AN AIRBORNE AGGREGATION PHEROMONE BLEND²

3.1 Abstract

Adults and juveniles of the common bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae) return to, and aggregate in, harbourages after host foraging. I tested the hypothesis that the aggregation is mediated, in part, by an airborne aggregation pheromone. Volatiles emanating from experimental *C. lectularius* harbourages were captured on Porapak Q columns, silica-fractionated and bioassayed in dual-choice, still-air olfactometer experiments. Of 14 compounds with threshold abundance in GC-MS analyses of two bioactive fractions, ten compounds [nonanal, decanal, (E)-2-hexenal, (E)-2-octenal, (E,E)-2,4-octadienal, benzaldehyde, (+)- and (−)-limonene, sulcatone and benzyl alcohol] proved to be essential components of the *C. lectularius* airborne aggregation pheromone. Synthetic aggregation pheromone lures in trapping devices could be developed for sensitive detection of *C. lectularius* in human dwellings.

3.2 Introduction

Previous studies have demonstrated that *C. lectularius* produce both an alarm pheromone consisting of (E)-2-hexenal and (E)-2-octenal, and an assembling scent (Marx 1955; Levinson and Bar Ilan 1971; Levinson et al. 1974a,b). The ‘assembling scent’ is only perceived by contact chemoreception (Chapter 2) and has not yet been identified.

² A modified version of this chapter has been submitted to the Journal of Chemical Ecology: Siljander, E., Gries, R., and Gries, G. Identification of the airborne aggregation pheromone of the common bed bug, *Cimex lectularius*. 
Cimex lectularius is known to aggregate within refugia, and will return to these harbourages after each blood meal (Kemper 1936). This behaviour has been attributed to a specific ‘nest odour’ (Marx 1955). However, there is no reported experimental evidence in support of an airborne aggregation pheromone. In the kissing bug, Triatoma infestans, both airborne attractant and chemotactic arrestant pheromones mediate aggregation behaviour (Schofield and Patterson 1977; Lorenzo Figueiras et al. 1994; Lorenzo and Lazzari 1996; Lorenzo Figueiras and Lazzari 1998). Likewise, C. lectularius might utilize both contact and airborne aggregation pheromones within the enclosed microhabitats in which they seek shelter.

In this chapter I show that C. lectularius use an airborne aggregation pheromone and reveal the pheromone components.

### 3.3 Materials and Methods

#### 3.3.1 Experimental Insects

Cimex lectularius were supplied by, or collected from, three sources: (1) Harold Harlan, Crownsville, MD, USA; (2) George Keeney, Ohio State University, Columbus, OH, USA; and (3) natural populations, Vancouver, BC, Canada. Insects were kept in wide-mouth glass jars (= colony jars) (9 x 9.5 cm) containing strips of cardboard folded accordion-style to provide a surface on which they could walk and oviposit. Insects were reared at 21-28 °C, 25-50 % R.H., and a 16L:8D photoperiod. They were blood-fed through an artificial membrane (Montes et al. 2002) or on human volunteers. The membrane system used heparinated chicken blood as a food source. For feeding on
humans, groups of insects were placed in jars (3.5 × 5.5 cm) topped with nylon mesh and allowed to feed to repletion.

3.3.2 Acquisition of Volatiles

To acquire airborne semiochemicals for bioassays and chemical analyses, colony jars containing 500-700 *C. lectularius* of mixed stage and gender were placed in a cylindrical Pyrex glass chamber (15.5 × 26 cm). In alternate volatile acquisitions, 100 adult male, 100 adult female, 100 juvenile or no *C. lectularius* were placed in separate cylindrical Pyrex glass chambers (10 × 18 cm). An electrical pump drew charcoal-filtered air at ~ 2 L/min through each chamber and through a glass column (14 × 1.3 cm OD) containing Porapak Q (50-80 mesh; Waters Associates, Inc., Milford, MA, USA). After 72 hr, volatiles were eluted from the Porapak Q trap with 2 ml of pentane. Jar aeration extracts (JAEs) contained ~ 43,200 bug hour equivalents (1 BHE = volatiles released by one *C. lectularius* during 1 hr), and were stored in darkness at -15°C.

3.3.3 Analytical Methods

Aliquots of JAEs were analyzed by coupled gas chromatography – mass spectrometry (GC-MS) in full-scan electron impact mode, using a Varian Saturn 2000 Ion Trap GC-MS fitted with a DB-5 column (30 m × 0.25 mm ID, JandW Scientific, Folsom, CA, USA). The temperature program started at 50°C for 2 min, and then increased at a rate of 10°C per min to 280°C. All volatile constituents of > 100 pg abundance were considered candidate pheromone components and were identified by their mass spectra, retention indices (Van den Dool and Kratz 1963) and by comparison with authentic standards.
Aliquots of JAE were concentrated under a nitrogen stream and fractionated through silica gel (0.5 g) in a glass column (14 × 0.5 cm ID). After pre-rinsing the silica with pentane, JAE aliquots were applied, allowed to impregnate the silica, and then eluted with five consecutive rinses (2 ml each) of pentane/ether, with increasing proportion of ether, as follows: (1) 100:0; (2) 90:10; (3) 75:25; (4) 50:50; (5) 0:100. This procedure generated five fractions containing analytes of increasing polarity.

3.3.4 Olfactometer Experiments

Responses of *C. lectularius* to test stimuli were bioassayed in still-air olfactometers because *C. lectularius* inhabit enclosed microhabitats with little or no air movement and avoid moving air (Kemper 1936; Johnson 1941; Usinger 1966; personal observation). The olfactometers consisted of three glass chambers (each 3.5 × 10 cm ID) linearly interconnected by glass tubes (each 2.5 × 1 cm ID) (Takács and Gries 2001). Test stimuli were randomly assigned to one of the two lateral chambers, each containing a paper towel disc (9 cm diam.; folded eight times for corrugation) with a tab (2.5 × 0.7 cm) extending through to the central chamber to facilitate inter-chamber movement of bioassay insects. Stimuli were pipetted onto the discs, with treatment and control discs receiving the same volume of solvent. For each of 23-50 replicates in each of experiments 1-44 (Table 1), one naive *C. lectularius* was released in the central chamber of the olfactometer three hours before the end of scotophase, when *C. lectularius* is most active (Mellanby 1939b). An insect was classed as a responder when it was found 16-18 hr later on a paper disc or within a lateral glass tube in a state of akinesis. To rigorously compare the effectiveness of test stimuli, experiments were often run in parallel to minimize the effect of any lurking variables, such as barometric pressure or time since
**Table 3.1** Summary of experimental insects and stimuli tested in laboratory still-air and cage bioassays.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Treatment Stimulus</th>
<th>Control Stimulus</th>
<th>Responder</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Porapak Q extract at 2000 BHE&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>Solvent&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Adult ♀</td>
<td>43</td>
</tr>
<tr>
<td>2</td>
<td>2000 BHE&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Solvent</td>
<td>Adult ♀</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>2000 BHE&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>100 BHE&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>200 BHE</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>36</td>
</tr>
<tr>
<td>6</td>
<td>All 5 silica gel fractions recombined</td>
<td>Solvent&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Juvenile</td>
<td>30</td>
</tr>
<tr>
<td>7</td>
<td>All minus fraction 1</td>
<td>Solvent&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Juvenile</td>
<td>30</td>
</tr>
<tr>
<td>8</td>
<td>All minus fraction 2</td>
<td>Solvent&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Juvenile</td>
<td>30</td>
</tr>
<tr>
<td>9</td>
<td>All minus fraction 3</td>
<td>Solvent&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Juvenile</td>
<td>30</td>
</tr>
<tr>
<td>10</td>
<td>All minus fraction 4</td>
<td>Solvent&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Juvenile</td>
<td>30</td>
</tr>
<tr>
<td>11</td>
<td>All minus fraction 5</td>
<td>Solvent&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Juvenile</td>
<td>30</td>
</tr>
<tr>
<td>12</td>
<td>Synthetic Blend&lt;sup&gt;e&lt;/sup&gt; (SB)</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>36</td>
</tr>
<tr>
<td>13</td>
<td>SB</td>
<td>Solvent</td>
<td>Adult ♂</td>
<td>45</td>
</tr>
<tr>
<td>14</td>
<td>SB</td>
<td>Solvent</td>
<td>Mated Adult ♀</td>
<td>45</td>
</tr>
<tr>
<td>15</td>
<td>SB</td>
<td>Solvent</td>
<td>Virgin Adult ♀</td>
<td>36</td>
</tr>
<tr>
<td>16</td>
<td>SB</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>23</td>
</tr>
<tr>
<td>17</td>
<td>SB minus monoterpenes</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>24</td>
</tr>
<tr>
<td>18</td>
<td>SB minus benzyl derivatives</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>23</td>
</tr>
<tr>
<td>19</td>
<td>SB minus ketones</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>23</td>
</tr>
<tr>
<td>20</td>
<td>SB minus saturated aldehydes</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>23</td>
</tr>
<tr>
<td>21</td>
<td>SB minus monoene-aldehydes</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>23</td>
</tr>
<tr>
<td>22</td>
<td>SB minus diene-aldehydes</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>23</td>
</tr>
<tr>
<td>23</td>
<td>SB</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>33</td>
</tr>
<tr>
<td>24</td>
<td>SB minus (E)-2-hexenal</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>32</td>
</tr>
<tr>
<td>25</td>
<td>SB minus benzaldehyde</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>31</td>
</tr>
<tr>
<td>26</td>
<td>SB minus sulcatone</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>33</td>
</tr>
<tr>
<td>27</td>
<td>SB minus octanal</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>32</td>
</tr>
<tr>
<td>28</td>
<td>SB minus (+)-limonene</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>35</td>
</tr>
<tr>
<td>29</td>
<td>SB minus (−)-limonene</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>32</td>
</tr>
<tr>
<td>30</td>
<td>SB minus benzyl alcohol</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>32</td>
</tr>
<tr>
<td>31</td>
<td>SB minus (E)-2-octenal</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>30</td>
</tr>
<tr>
<td>32</td>
<td>SB minus (E,Z)-2,4-octadienal</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>32</td>
</tr>
<tr>
<td>33</td>
<td>SB minus nonanal</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>32</td>
</tr>
<tr>
<td>34</td>
<td>SB minus benzyl acetate</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>33</td>
</tr>
<tr>
<td>35</td>
<td>SB minus decanal</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>30</td>
</tr>
<tr>
<td>36</td>
<td>SB minus geranylacetone</td>
<td>Solvent</td>
<td>Juvenile</td>
<td>33</td>
</tr>
</tbody>
</table>
### Still-air olfactometer bioassays

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Treatment</th>
<th>Control Stimulus</th>
<th>Responder</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>New Synthetic Blend(^f) (NSB)</td>
<td>Solvent</td>
<td>Juvenile</td>
</tr>
<tr>
<td>38</td>
<td>NSB (\text{minus} (E,Z)-2,4)-octadienal</td>
<td>Solvent</td>
<td>Juvenile</td>
</tr>
<tr>
<td>39</td>
<td>NSB (\text{minus} \text{ benzyl acetate}</td>
<td>Solvent</td>
<td>Juvenile</td>
</tr>
<tr>
<td>40</td>
<td>NSB (\text{minus} \text{ geranylacetone}</td>
<td>Solvent</td>
<td>Juvenile</td>
</tr>
<tr>
<td>41</td>
<td>NSB (\text{minus} (E,Z)-2,4)-octadienal (\text{minus benzyl acetate}</td>
<td>Solvent</td>
<td>Juvenile</td>
</tr>
<tr>
<td>42</td>
<td>NSB (\text{minus} (E,Z)-2,4)-octadienal (\text{minus geranylacetone}</td>
<td>Solvent</td>
<td>Juvenile</td>
</tr>
<tr>
<td>43</td>
<td>NSB (\text{minus} \text{ benzyl acetate}</td>
<td>Solvent</td>
<td>Juvenile</td>
</tr>
<tr>
<td>44</td>
<td>NSB (\text{minus} (E,Z)-2,4)-octadienal (\text{minus benzyl acetate}</td>
<td>Solvent</td>
<td>Juvenile</td>
</tr>
</tbody>
</table>

### Cage bioassays

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Treatment</th>
<th>Control</th>
<th>Responders</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>10 SB</td>
<td>Solvent</td>
<td>10 Juveniles</td>
</tr>
<tr>
<td>46</td>
<td>10 SB (\text{plus} \text{ Juvenile contact pheromone (720 CPHE)}</td>
<td>Solvent (\text{plus} \text{ Juvenile contact pheromone (720 CPHE)}</td>
<td>10 Juveniles</td>
</tr>
</tbody>
</table>

\(^a\) Still-air olfactometers not placed in plastic bins for duration of bioassay;  
\(^b\) BHE = bug hour equivalents; 1 BHE = volatiles released by one \(C.\text{lectularius}\) during 1 hr;  
\(^c\) In all experiments, treatment and control stimuli received the same amount and composition of solvent(s);  
\(^d\) Solvent consisted of a mixture of pentane (60%) and ether (40%);  
\(^e\) SB = synthetic blend of 14 components: octanal (0.6 ng), nonanal (3.6 ng), decanal (2.0 ng), \((E)\)-2-hexenal (18.4 ng), \((E)\)-2-octenal (32.1 ng), \((E,E)\)-2,4-octadienal (0.4 ng), \((E,Z)\)-2,4-octadienal (0.3 ng), benzaldehyde (0.7 ng), (+)-limonene (0.9 ng), (−)-limonene (0.9 ng), sulcatone (0.4 ng), geranylacetone (2.4 ng), benzyl alcohol (0.6 ng);  
\(^f\) NSB = new synthetic blend = NSB \(\text{minus} \text{ octanal} ;  
\(^g\) CPHE = contact pheromone hour equivalents; 1 CPHE = contact pheromone deposited by one \(C.\text{lectularius}\) during 1 hr.

Note: Experiments grouped by brackets were run in parallel.
last feeding. All experiments were conducted at 22-24 °C, 25-40 % R.H., under an 18-hr photoperiod of 3D:15L. After each replicate, olfactometers were washed thoroughly with hot water and Sparklene™ detergent and were oven-dried at 125°C for 3 hr.

To determine whether the JAE was attractive, experiments 1-3 tested the responses of adult males, adult females, and juveniles (4th or 5th instar) to 10 JAE (≈ 2000 BHE). Experiment 4 tested the effect of a lower dose (100 BHE) on the response of juveniles. In experiment 5, olfactometers were stacked (three rows × three columns) inside yellow plastic bins (38 × 31 × 11 cm; Columbia Plastics Ltd.®, Vancouver, BC), to determine whether no moving air and no directional light improved the response of juveniles to ~ 200 BHE. All subsequent still-air olfactometer experiments were conducted according to this protocol.

To determine candidate pheromone components, parallel-run experiments 6-11 tested the five recombined silica gel fractions of JAE vs. blends lacking single fractions. Taking the results of experiments 6-11 and those of GC-MS analyses into account, experiments 12-15 tested a synthetic blend (SB) of 14 candidate pheromone components [octanal, nonanal, decanal, (E)-2-hexenal, (E)-2-octenal, (E,E)-2,4-octadienal, (E,2)-2,4-octadienal, benzaldehyde, benzyl alcohol, benzyl acetate, (+)-limonene, (-)-limonene, sulcatone, geranylacetone] at 200 BHE for its ability to attract juveniles, adult males, mated adult females, and virgin adult females.

To identify essential components in the SB, parallel-run experiments 16-22 tested SB vs. blends lacking groups of organic chemicals, such as monoterpenes (experiment 17), benzyl derivatives (experiment 18), ketones (experiment 19), saturated aldehydes (experiment 20), monoene-aldehydes (experiment 21), or diene-aldehydes (experiment
22). Similarly, parallel-run experiments 23-36 tested SB vs. blends lacking a single component. (E,E)-2,4-Octadienal could not be removed as a single component because it kept forming as a rearrangement product of (E,Z)-2,4-octadienal or could not be sufficiently isolated by high-performance liquid chromatography. Considering the results of experiments 23-36, parallel-run experiments 37-44 tested a new synthetic blend (NSB) [SB minus octanal] vs. blends lacking one, two or all of the three components (E,Z)-2,4-octadienal, benzyl acetate and geranylacetone.

In experiment 45 the ability of the 14-component SB to attract or arrest C. lectularius was tested in Plexiglas cage (30 × 30 × 42 cm) olfactometers, with four mesh-covered holes (18 cm diam.) for ventilation and a sliding door (21 × 25 cm) for access. The textured (sandpaper-treated) cage floor allowed the insects to walk easily to either of two paper disc shelters (6 cm diam.; folded six times for corrugation) in opposing corners of the cage. The treatment stimulus (10 SB ≈ 2000 BHE) and solvent control stimulus were randomly assigned and pipetted onto these discs, with treatment and control discs receiving the same volume of solvent. For each of 10 replicates in experiments 45-46 (Table 3.1), 10 naive juveniles were released in the center of the cage three hours before the end of scotophase, and the number of juveniles on each disc was recorded 16-18 hr later. Insects were classed as responders when they were found on a paper disc in a state of akinesia. All experiments were conducted at 22-24 °C, 25-40 % R.H., under an 18-hr photoperiod of 3D:15L. After each replicate, the cages were sequentially washed with 70% ethanol and hexane, and then dried for 5 hr.
Follow-up experiment 46 tested whether the response of insects to airborne pheromone in experiment 45 could be enhanced by adding juvenile contact pheromone (Chapter 2) at 720 CPHE to both treatment and control discs.

### 3.3.5 Statistical Analyses

Numbers of *C. lectularius* responding to treatment and control stimuli in 3-chamber olfactometer experiments 1-44 were analyzed with the $\chi^2$ goodness-of-fit test, using Yates correction for continuity ($\alpha = 0.05$) (Zar 1999). The mean proportions of juvenile *C. lectularius* responding to treatment and control stimuli in cage olfactometer experiments 45-46 were analyzed with the Wilcoxon paired-sample test ($\alpha = 0.05$) (Zar 1999). Non-responders were not included in statistical analyses.

### 3.4 Results

Porapak Q colony jar aeration extracts (JAEs) tested at 2000 BHE attracted/arrested adult females and juveniles (Figure 3.1, experiments 2-3) but failed to elicit a significant response from adult males (Figure 3.1, experiment 1). The lower dose of 100 BHE was still significantly effective for juveniles (Figure 3.1, experiment 4). When olfactometers were housed within plastic bins, juveniles strongly responded to 200 BHE (Figure 3.2, experiment 5).

A recombination of all five silica gel fractions of JAE elicited significant responses from juveniles, as did blends lacking fractions 1, 3 or 5 (Figure 3.2, experiments 6, 7, 9, 11). JAE blends lacking either fraction 2 or 4 had no significant behavioural activity (Figure 3.2, experiments 8, 10), suggesting that fractions 2 and 4 contained most, if not all, bioactive components. These components were identified by
Figure 3.1 Response of adult male (experiment 1), adult female (experiment 2) and juvenile (experiments 3, 4) *Cimex lectularius* in still-air olfactometers to aliquots [100 or 2000 bug hour equivalents (BHE)] of Porapak Q colony jar aeration extracts. 1 BHE = volatiles released by one *C. lectularius* during 1 hr. Number of insects responding to each stimulus is given within bars; number of insects not responding in each experiment is given in parentheses. For each experiment, *P*-values are provided in the figure; $\chi^2$ test with Yates correction for continuity; $\alpha = 0.05$. The same volume of pentane (7.5 µl) was applied to treatment and control stimuli.
Exp. 1
2000 BHE 25 (4)  
Solvent control 14  
Adult respondents

Exp. 2
2000 BHE 31 (3)  
Solvent control 16  
Adult respondents

Exp. 3
2000 BHE 28 (1)  
Solvent control 11  
Juvenile respondents

Exp. 4
100 BHE 26 (5)  
Solvent control 9  
Juvenile respondents

Test stimuli  Number of *C. lectularius* responding

$P = 0.109$

$P = 0.041$

$P = 0.010$

$P = 0.007$
Figure 3.2  Response of juvenile *Cimex lectularius* in still-air olfactometers to aliquots (200 BHE; see Figure 3.1) of Porapak Q colony jar aeration extracts (experiment 5), or to silica gel fractions (200 BHE) of the same extract (experiments 6-11). Number of insects responding to each stimulus is given within bars; number of insects not responding in each experiment is given in parentheses. For each experiment, *P*-values are provided in the figure; \(\chi^2\) test with Yates correction for continuity; \(\alpha = 0.05\). Experiments 6-11 were run in parallel; the same volume of pentane (10 µl, experiment 5; 50 µl, experiments 6-11) was applied to treatment and control stimuli.
## Experimental Results

### Exp. 5

<table>
<thead>
<tr>
<th>Test Stimulus</th>
<th>Number of Juvenile C. lectularius Responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 BHE</td>
<td>30 (3)</td>
</tr>
<tr>
<td>Solvent control</td>
<td>3</td>
</tr>
</tbody>
</table>

### Exp. 6

<table>
<thead>
<tr>
<th>Test Stimulus</th>
<th>Number of Juvenile C. lectularius Responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>All 5 fractions recombined</td>
<td>18 (7)</td>
</tr>
<tr>
<td>Solvent control</td>
<td>5</td>
</tr>
</tbody>
</table>

### Exp. 7

<table>
<thead>
<tr>
<th>Test Stimulus</th>
<th>Number of Juvenile C. lectularius Responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>All minus fraction 1</td>
<td>19 (7)</td>
</tr>
<tr>
<td>Solvent control</td>
<td>4</td>
</tr>
</tbody>
</table>

### Exp. 8

<table>
<thead>
<tr>
<th>Test Stimulus</th>
<th>Number of Juvenile C. lectularius Responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>All minus fraction 2</td>
<td>12 (14)</td>
</tr>
<tr>
<td>Solvent control</td>
<td>4</td>
</tr>
</tbody>
</table>

### Exp. 9

<table>
<thead>
<tr>
<th>Test Stimulus</th>
<th>Number of Juvenile C. lectularius Responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>All minus fraction 3</td>
<td>17 (10)</td>
</tr>
<tr>
<td>Solvent control</td>
<td>3</td>
</tr>
</tbody>
</table>

### Exp. 10

<table>
<thead>
<tr>
<th>Test Stimulus</th>
<th>Number of Juvenile C. lectularius Responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>All minus fraction 4</td>
<td>13 (10)</td>
</tr>
<tr>
<td>Solvent control</td>
<td>7</td>
</tr>
</tbody>
</table>

### Exp. 11

<table>
<thead>
<tr>
<th>Test Stimulus</th>
<th>Number of Juvenile C. lectularius Responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>All minus fraction 5</td>
<td>18 (8)</td>
</tr>
<tr>
<td>Solvent control</td>
<td>4</td>
</tr>
</tbody>
</table>
comparing their GC-retention times and mass spectra with those of authentic standards.
The 14 candidate pheromone components were octanal, nonanal, decanal, \((E)-2\)-hexenal, \((E)-2\)-octenal, \((E,E)-2,4\)octadienal, \((E,Z)-2,4\)octadienal, benzaldehyde, benzyl alcohol, benzyl acetate, \((+)\)-limonene, \((-)\)-limonene, sulcatone and geranylacetone. A synthetic blend (SB) of these 14 candidate pheromone components tested at 200 BHE elicited significant behaviour responses from juveniles, adult males and virgin adult females (Figure 3.3, experiments 12, 13, 15) but not from mated adult females (Figure 3.3, experiment 14). SBs lacking monoterpenes, benzyl derivatives, ketones, saturated aldehydes, monoene-aldehydes, or diene-aldehydes were not behaviourally active (Figure 3.4, experiments 16-22). SBs lacking either octanal, \((E,Z)-2,4\)-octadienal, benzyl acetate or geranylacetone remained behaviourally active (Figure 3.5, experiments 27, 32, 34, 36), whereas SBs lacking either \((E)-2\)-hexenal, benzaldehyde, sulcatone, \((+)\)-limonene, \((-)\)-limonene, benzyl alcohol, \((E)-2\)-octenal, nonanal, or decanal did not elicit significant behaviour responses (Figure 3.5, experiments 24-26, 28-31, 33, 35). A new synthetic blend (NSB) \([SB \text{ minus octanal}]\) failed to affect juveniles (Figure 3.6, experiment 37) and NSBs lacking \((E,Z)-2,4\)-octadienal, benzyl acetate, or both, were not behaviourally active (Figure 3.6, experiments 38, 39, 41). All NSBs lacking geranylacetone, including the NSB without the four components octanal, \((E,Z)-2,4\)-octadienal, benzyl acetate and geranylacetone, elicited significant behaviour responses (Figure 3.6, experiments 40, 42-44).

In cage experiment 45, more juveniles were found on discs treated with SB at 2000 BHE than on control discs, but this result was not statistically significant (Figure 3.7). In cage experiment 46, SB tested in combination with juvenile contact pheromone at
Figure 3.3 Response of juvenile (experiment 12), adult male (experiment 13), mated adult female (experiment 14) and virgin adult female (experiment 15) *Cimex lectularius* in still-air olfactometers to a synthetic blend (SB) of 14 candidate pheromone components at 200 BHE. Number of insects responding to each stimulus is given within bars; number of insects not responding in each experiment is given in parentheses. For each experiment, $P$-values are provided in the figure; $\chi^2$ test with Yates correction for continuity; $\alpha = 0.05$.

The SB consisted of three saturated aldehydes [octanal, nonanal, decanal], four unsaturated aldehydes [(E)-2-hexenal, (E)-2-octenal, (E,E)-2,4-octadienal, (E,Z)-2,4-octadienal], one aromatic aldehyde [benzaldehyde], two monoterpenes [(+)-limonene, (−)-limonene], two ketones [sulcatone, geranylacetone], one acetate [benzyl acetate] and one aromatic alcohol [benzyl alcohol]. The same volume of pentane (10-15 μl) was applied to treatment and control stimuli.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Condition</th>
<th>Test Stimuli</th>
<th>Number of C. lectularius Responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 12</td>
<td>Synthetic blend (SB)</td>
<td>0, 5, 10, 15, 20, 25, 30</td>
<td>6, 10, 18, 24, 27</td>
</tr>
<tr>
<td></td>
<td>Solvent control</td>
<td></td>
<td>0, 5, 10, 15, 20, 25, 30</td>
</tr>
<tr>
<td>Exp. 13</td>
<td>SB</td>
<td>0, 5, 10, 15, 20, 25, 30</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Solvent control</td>
<td></td>
<td>0, 5, 10, 15, 20, 25, 30</td>
</tr>
<tr>
<td>Exp. 14</td>
<td>SB</td>
<td>0, 5, 10, 15, 20, 25, 30</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Solvent control</td>
<td></td>
<td>0, 5, 10, 15, 20, 25, 30</td>
</tr>
<tr>
<td>Exp. 15</td>
<td>SB</td>
<td>0, 5, 10, 15, 20, 25, 30</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Solvent control</td>
<td></td>
<td>0, 5, 10, 15, 20, 25, 30</td>
</tr>
</tbody>
</table>

*Juvenile responders: P < 0.001 (3)  
Adult responders: P = 0.026 (11)  
Mated adult responders: P = 0.596 (13)  
Virgin adult responders: P = 0.022 (4)
Figure 3.4 Response of juvenile *Cimex lectularius* in still-air olfactometer experiments 16-22 to synthetic blends (SB) at 200 BHE, lacking groups of candidate pheromone components. Number of insects responding to each stimulus is given within bars; number of insects not responding in each experiment is given in parentheses. For each experiment, $P$-values are provided in the figure; $\chi^2$ test with Yates correction for continuity; $\alpha = 0.05$. All experiments were run in parallel; the same volume of pentane (15 $\mu$l) was applied to treatment and control stimuli.
<table>
<thead>
<tr>
<th>Exp. 16</th>
<th>Synthetic blend (SB)</th>
<th>18 (1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent control</td>
<td>4</td>
<td>$P = 0.006$</td>
</tr>
<tr>
<td>Exp. 17</td>
<td>SB minus monoterpenes</td>
<td>13 (2)</td>
</tr>
<tr>
<td>Solvent control</td>
<td>9</td>
<td>$P = 0.522$</td>
</tr>
<tr>
<td>Exp. 18</td>
<td>SB minus benzyl derivatives</td>
<td>15 (1)</td>
</tr>
<tr>
<td>Solvent control</td>
<td>7</td>
<td>$P = 0.136$</td>
</tr>
<tr>
<td>Exp. 19</td>
<td>SB minus ketones</td>
<td>9 (3)</td>
</tr>
<tr>
<td>Solvent control</td>
<td>11</td>
<td>$P = 0.823$</td>
</tr>
<tr>
<td>Exp. 20</td>
<td>SB minus saturated aldehydes</td>
<td>12 (2)</td>
</tr>
<tr>
<td>Solvent control</td>
<td>9</td>
<td>$P = 0.663$</td>
</tr>
<tr>
<td>Exp. 21</td>
<td>SB minus monoene-aldehydes</td>
<td>12 (1)</td>
</tr>
<tr>
<td>Solvent control</td>
<td>10</td>
<td>$P = 0.831$</td>
</tr>
<tr>
<td>Exp. 22</td>
<td>SB minus diene-aldehydes</td>
<td>13 (0)</td>
</tr>
<tr>
<td>Solvent control</td>
<td>10</td>
<td>$P = 0.677$</td>
</tr>
</tbody>
</table>

Test stimuli | Number of juvenile *C. lectularius* responding
Figure 3.5 Response of juvenile *Cimex lectularius* in still-air olfactometer experiments 23-36 to synthetic blends (SB) at 200 BHE, lacking single candidate pheromone components. Number of insects responding to each stimulus is given within bars; number of insects not responding in each experiment is given in parentheses. For each experiment, *P*-values are provided in the figure; \( \chi^2 \) test with Yates correction for continuity; \( \alpha = 0.05 \). All experiments were run in parallel; the same volume of pentane (15 \( \mu \)l) was applied to treatment and control stimuli.
| Exp. 23 | Synthetic blend (SB) | Solvent control | 22 | (3) | P = 0.018 |
| Exp. 24 | SB minus (E)-2-hexenal | Solvent control | 19 | (2) | P = 0.201 |
| Exp. 25 | SB minus benzaldehyde | Solvent control | 17 | (1) | P = 0.584 |
| Exp. 26 | SB minus sulcatone | Solvent control | 19 | (3) | P = 0.201 |
| Exp. 27 | SB minus octanal | Solvent control | 24 | (2) | P = 0.002 |
| Exp. 28 | SB minus (+)-limonene | Solvent control | 19 | (5) | P = 0.201 |
| Exp. 29 | SB minus (-)-limonene | Solvent control | 19 | (3) | P = 0.137 |
| Exp. 30 | SB minus benzyl alcohol | Solvent control | 18 | (3) | P = 0.265 |
| Exp. 31 | SB minus (E)-2-octenal | Solvent control | 17 | (0) | P = 0.584 |
| Exp. 32 | SB minus (E,Z)-2,4-octadienal | Solvent control | 21 | (2) | P = 0.045 |
| Exp. 33 | SB minus nonanal | Solvent control | 17 | (3) | P = 0.458 |
| Exp. 34 | SB minus benzyl acetate | Solvent control | 21 | (3) | P = 0.045 |
| Exp. 35 | SB minus decanal | Solvent control | 12 | (0) | P = 0.361 |
| Exp. 36 | SB minus geranylacetone | Solvent control | 21 | (3) | P = 0.045 |

Test stimuli: Synthetic blend (SB) and solvent control. Number of juvenile C. lectularius responding.
**Figure 3.6** Response of juvenile *Cimex lectularius* in still-air olfactometer experiments 37-44 to new synthetic blends (NSB; blend as described in caption of Figure 3.3 without octanal) at 200 BHE, lacking one or more candidate pheromone components. Number of insects responding to each stimulus is given within bars; number of insects not responding in each experiment is given in parentheses. For each experiment, *P*-values are provided in the figure; $\chi^2$ test with Yates correction for continuity; $\alpha = 0.05$. All experiments were run in parallel; the same volume of pentane (10 μl) was applied to treatment and control stimuli.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Test stimuli</th>
<th>Number of juvenile <em>C. lectularius</em> responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 37</td>
<td>New Synthetic Blend (NSB)</td>
<td>18 (3)</td>
</tr>
<tr>
<td></td>
<td>Solvent control</td>
<td>13</td>
</tr>
<tr>
<td>Exp. 38</td>
<td>NSB minus (E,Z)-2,4-octadienal</td>
<td>18 (2)</td>
</tr>
<tr>
<td></td>
<td>Solvent control</td>
<td>14</td>
</tr>
<tr>
<td>Exp. 39</td>
<td>NSB minus benzyl acetate</td>
<td>21 (2)</td>
</tr>
<tr>
<td></td>
<td>Solvent control</td>
<td>11</td>
</tr>
<tr>
<td>Exp. 40</td>
<td>NSB minus geranylacetone</td>
<td>21 (4)</td>
</tr>
<tr>
<td></td>
<td>Solvent control</td>
<td>9</td>
</tr>
<tr>
<td>Exp. 41</td>
<td>NSB minus (E,Z)-2,4-octadienal minus benzyl acetate</td>
<td>21 (0)</td>
</tr>
<tr>
<td></td>
<td>Solvent control</td>
<td>13</td>
</tr>
<tr>
<td>Exp. 42</td>
<td>NSB minus (E,Z)-2,4-octadienal minus geranylacetone</td>
<td>25 (4)</td>
</tr>
<tr>
<td></td>
<td>Solvent control</td>
<td>5</td>
</tr>
<tr>
<td>Exp. 43</td>
<td>NSB minus benzyl acetate minus geranylacetone</td>
<td>23 (1)</td>
</tr>
<tr>
<td></td>
<td>Solvent control</td>
<td>9</td>
</tr>
<tr>
<td>Exp. 44</td>
<td>NSB minus (E,Z)-2,4-octadienal minus benzyl acetate minus geranylacetone</td>
<td>21 (4)</td>
</tr>
<tr>
<td></td>
<td>Solvent control</td>
<td>8</td>
</tr>
</tbody>
</table>

Test stimuli | Number of juvenile *C. lectularius* responding

59
Figure 3.7 Mean (+ SE) proportion of 10 juvenile *Cimex lectularius* per replicate (10 replicates) responding in cage olfactometer experiments 45 and 46 to a synthetic blend (SB; blend composition as described in caption of Figure 3.3) at 2000 BHE (experiment 45) or SB in combination with *C. lectularius* juvenile contact pheromone at 720 contact pheromone hour equivalents (CPHE) (experiment 46). 1 CPHE = contact pheromone deposited by one *C. lectularius* during 1 hr. For each experiment, *P*-values are provided in the figure; Wilcoxon paired-sample test; \( \alpha = 0.05 \). The same volume of pentane (50 \( \mu l \)) for SB application or methanol (100 \( \mu l \)) for CPHE application was applied to treatment and control stimuli.
Exp. 45

- Synthetic Blend (SB)
- Solvent control

Test stimulus Mean (+ SE) proportion of juvenile *C. lectularius* responding

Exp. 46

- SB plus juvenile contact pheromone (720 CPHE)
- Solvent control plus juvenile contact pheromone (720 CPHE)

Test stimulus Mean (+ SE) proportion of juvenile *C. lectularius* responding

P > 0.050

P < 0.050
720 CPHE attracted/arrested a greater proportion of test insects than did contact pheromone alone (Figure 3.7).

Of the ten essential pheromone components, all except benzyl alcohol were detected by GC-MS in Porapak Q extracts of adult male, adult female and juvenile aerations. Quantities collected from juveniles were lower than from either adult males or females. (E)-2-Hexenal, (E)-2-octenal, (E,E)-2,4-octadienal and benzaldehyde were only detected when insects were present in the aeration chamber. Sulcatone, nonanal and decanal were detected in higher quantities when insects were present in the aeration chamber than in blank aeration controls. The quantities of limonene in aerations of adult males, but not in aerations of adult females or juveniles, were higher than those in blank controls.

3.5 Discussion

My data provide evidence that adult male, adult female and juvenile *C. lectularius* emit and respond to an airborne aggregation pheromone. With ten essential components, the pheromone is unusually complex. Such a complex aggregation pheromone has only been reported for cocoon-spinning *Cydia pomonella* larvae (Jumean et al. 2005). Similarly complex sex pheromones occur in 14 species of the Lepidoptera, each deploying pheromone blends of six or more components (Byers 2006).

The specific origin of each of the ten essential pheromone components remains unknown, but (E)-2-hexenal, (E)-2-octenal, (E,E)-2,4-octadienal, benzaldehyde, nonanal, decanal and sulcatone were all prevalent in headspace volatiles from adult male, adult female and juvenile *C. lectularius*. The trace component benzyl alcohol co-eluted with
limonene in GC-MS analyses, rendering its detection and quantification difficult. (+)-
and/or (-)-Limonene was present in above-background quantity in aeration of adult males, suggesting that males are the primary or sole emitters of limonene.

Paper towel discs served as release devices for JAEs and SBs in all olfactometer experiments. The concern that the relative ratio of synthetic pheromone components released from paper towel discs may differ from that of natural pheromone components released by insects, was addressed by comparative analyses of headspace volatiles from both sources. These analyses did not reveal a significant difference in the composition of natural and synthetic pheromone blend release.

The two most abundant aggregation pheromone components in *C. lectularius*, *(E)-2-hexenal and (E)-2-octenal*, have been previously reported as alarm pheromones (Levinson and Bar Ilan 1971; Levinson et al. 1974a,b). Their threshold for eliciting alarm behaviour [concentration measured at time of alarm reaction: 1.3-2.3 µg/ml air for *(E)-2-hexenal*; 0.25-0.29 µg/ml air for *(E)-2-octenal*], greatly exceeds the physiological detection threshold estimated to be $2 \times 10^{10}$ molecules/ml air for *(E)-2-hexenal* [3.25$\times 10^{-6}$ µg/ml air] (Levinson et al. 1974b). The detection threshold for *(E)-2-octenal* would be similar because it elicited receptor potentials at the same dose as *(E)-2-hexenal* (Levinson et al. 1974b). The levels tested in my study did not exceed 9.50$\times 10^{4}$ µg/ml air of *(E)-2-hexenal* or 1.56$\times 10^{3}$ µg/ml air of *(E)-2-octenal*, making them significantly lower than the alarm response threshold, but higher than the proposed physiological detection threshold. These data and calculations suggest that *(E)-2-hexenal* and *(E)-2-octenal* serve as multifunctional pheromone components that are attractive/arrestant at low concentrations but repellent at high concentrations (Borden 1985; Blum 1996). Multifunctional
pheromones also have been reported in other Hemiptera, such as *Eurydema rugosa* (Ishiwatari 1974, 1976) and *Nezara viridula* (Lockwood and Story 1985).

Volatile pheromones mediating aggregation behaviour may attract and/or arrest conspecifics. The still-air and cage olfactometer experiments did not allow me to distinguish clearly between attraction and arrestment responses. Anemotactic responses to pheromone would unambiguously demonstrate long-range attractiveness, but such responses would not likely be obtainable due to *C. lectularius* avoidance of moving air (Kemper 1936; Johnson 1941; Usinger 1966). However, results of cage experiments (Figure 3.7) imply that the aggregation pheromone functions mainly as an attractant. SB alone appeared to be insufficient to attract and arrest a significant proportion of juveniles (experiment 45), but when the juvenile-contact pheromone was added to both treatment and control discs, SB was preferred significantly over the solvent control (experiment 46). If the juvenile-contact pheromone were to arrest juveniles at the site of their first choice, then the airborne aggregation pheromone must have been an attractant. In natural settings, the airborne aggregation pheromone may function to direct refuge-searching *C. lectularius* towards established harbourages, whereas the stage-specific contact pheromones (Chapter 2) arrest them at the source.

Aggregation pheromones likely have evolved due to the Allee effect, which is a positive relationship between any component of individual fitness and either numbers or density of conspecifics (Stephens *et al.* 1999; Wertheim *et al.* 2005). The use of pheromones by *C. lectularius* to locate aggregations of conspecifics within refugia may benefit individuals by marking harbourages with suitable microclimates, potential mates and protection from predators. Aggregated juvenile *C. lectularius* may benefit from
increased desiccation tolerance and higher rate of development at lower temperatures, a phenomenon observed in nymphs of *N. viridula* (Lockwood and Story 1986). Locating aggregated conspecifics may also facilitate mate-finding for adult *C. lectularius*, which do not appear to possess a true sex pheromone to mediate mate attraction. Also, adults in aggregations may benefit from communal calling that increases the active space of pheromone and efficiency of mate-finding (Wertheim *et al.* 2005). Individual *C. lectularius* in aggregations also may benefit from increased vigilance for natural enemies, which is suggested by the co-occurrence of aggregation and alarm pheromones (Levinson and Bar Ilan 1971; Levinson *et al.* 1974a,b; Wertheim *et al.* 2005).

The pheromone elicited significant behavioural response from juveniles, adult males and virgin adult females (Figure 3.3; experiments 12, 13, 15). The fact that mated adult females did not respond to pheromone (Figure 3.3; experiment 14) might be attributed to the injurious nature of mating in *C. lectularius*. During traumatic insemination, the male pierces the abdomen of the female with a needle-like intromittent organ, the paramere, and releases sperm into the female’s body cavity (Carayon 1966). Females have a specialized structure (spermalege) that receives sperm and contributes to wound-healing and defense against infection, but frequent re-matings reduce longevity and reproductive success (Mellanby 1939a; Stutt and Siva-Jothy 2001; Morrow and Arnqvist 2003; Reinhardt *et al.* 2003). Considering that females may have little or no control over re-mating frequency (Stutt and Siva-Jothy 2001), gravid females may avoid repeated traumatic insemination by seeking new refugia devoid of conspecifics. Dispersal of gravid females would explain why most non-aggregated individuals in natural populations are females (Siljander, unpublished; R. Cooper, personal communication). It
would also explain the spread of populations over large distances. Wingless *C. lectularius* are reliant on host transportation from place to place on clothing, luggage or furniture (Usinger 1966; Doggett *et al.* 2004; Reinhardt and Siva-Jothy 2007). The likelihood of transportation on any of these objects depends upon *C. lectularius* actively seeking new refugia, as opposed to returning to the old one. Therefore, mated adult females not attracted to established harbourages may actively enter into passive dispersal more often than males or juveniles. This concept is supported by the findings that the majority of dispersing bat and bird bugs, detected on the bodies of host bats or birds, were adult females (Loye 1985; Heise 1988; Brown and Brown 2005; Reinhardt and Siva-Jothy 2007). Whether this concept applies to *C. lectularius* would require interception data (Doggett *et al.* 2004) which also reveal stage, gender and mating status of passively transported specimens.

With the airborne aggregation pheromone identified and shown to elicit behavioural responses in juveniles, adult males and adult females, there may be new opportunities to manipulate *C. lectularius* within infested dwellings. Synthetic pheromone could be placed in a trapping device to detect the presence of *C. lectularius* with greater sensitivity than current methods.
3.6 Reference List


CHAPTER 4
CONCLUDING SUMMARY

4.1 Conclusions

Haemtophagous Hemiptera employ multiple pheromones to mediate sexual, aggregative and alarm behaviour. In my thesis, I have investigated pheromonal communication in the common bed bug, *Cimex lectularius*.

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

1. Juvenile *C. lectularius* produce and deposit a contact pheromone on surfaces they frequently contact that arrests conspecific juveniles.

2. Juvenile-specific contact pheromone is, at least partially, extractable with methanol, yielding relatively effective pheromone extracts.

3. Adult male *C. lectularius* produce and deposit a contact pheromone on surfaces they frequently contact that arrests conspecific adult females and males.

4. Headspace volatiles from experimental harbourages of *C. lectularius* elicited significant responses from juveniles and adult females. Adult males also appeared to respond, but the results were not statistically significant.

5. A synthetic blend consisting of 14 candidate airborne pheromone components [octanal, nonanal, decanal, (E)-2-hexenal, (E)-2-octenal, (E,E)-2,4-octadienal, (E,Z)-2,4-octadienal, benzaldehyde, benzyl alcohol, benzyl acetate, (+)-limonene, (−)-
limonene, sulcatone and geranylacetone] elicited significant responses from juvenile, adult male and virgin adult female *C. lectularius*.

6. Of the 14 blend constituents (see # 5), the following ten proved to be essential pheromone components: nonanal, decanal, (E)-2-hexenal, (E)-2-octenal, (E,E)-2,4-octadienal, benzaldehyde, benzyl alcohol, (+)-limonene, (−)-limonene and sulcatone and elicited significant behavioural responses from juvenile *C. lectularius*.

7. All ten components, except for benzyl alcohol, were detected in aerations of adult male, adult female and juvenile *C. lectularius*, providing evidence that they are constituents of an airborne aggregation pheromone.

8. In natural settings the airborne aggregation pheromone may function to direct refuge searching *C. lectularius* towards established harbourages, whereas the stage-specific contact pheromones arrest them at the source.

9. Aggregation pheromones in *C. lectularius* might have evolved because individuals in aggregations may benefit from increased desiccation tolerance, higher rate of development, facilitated mate-finding and increased protection from predators.

### 4.2 Implications for Pest Management

Synthetic airborne aggregation pheromone, possibly in combination with male- and juvenile-specific contact pheromones, could be deployed within a retaining device to attract and capture *C. lectularius*. A bait- or capture-station could be placed in a room to potentially:
i. Detect the presence of *C. lectularius*;

ii. Divert them from other attractive sources such as sleeping humans;

iii. Eradicate an existing *C. lectularius* infestation; or

iv. Monitor for *C. lectularius* after insecticide applications for evaluation of treatment efficacy.

Field evaluations of the synthetic *C. lectularius* aggregation pheromone are required to determine the potential for its use in any of these contexts.