UNIVERSITY OF CALIFORNIA
RIVERSIDE

Odor-Mediated Aggregations of the Aposematic Coccinellid Beetle, *Hippodamia convergens*: *Supplementary Functions in Chemical Communication*

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

In

Evolution, Ecology, and Organismal Biology

By

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December 2013

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Acknowledgments

I would foremost like to thank my major advisor, Dr. Ring Cardé, without whom this dissertation would not have been possible. Throughout my graduate career he has provided the support and guidance integral to the success of my research. As an advisor, he has been able to strike the balance between proper mentorship while granting me necessary degree of independence. He urged me to pursue a research path new and independent to those typical of his lab, while still providing the necessary facilities and support.

I am thankful to my dissertation committee members, Drs. David Reznick, Jocelyn Millar, and Ring Cardé. The guidance they provided, from their various areas of expertise, was instrumental in facilitating my development as a scientist. Numerous components of this dissertation were conducted in close collaboration with Dr. Jocelyn Millar. I am immensely thankful for the time and effort he committed to the success of this project. I would also like to thank members of my qualifying exams committee, Drs. Kurt Anderson, Daphne Fairbairn, Dan Hare, and Rick Redak, who helped me define my research goals and advanced me to Ph.D. candidacy. And a special thanks to the other members of my lab, Nageen Asadi, Sima Asadi, Ian Hudson, Kim Klinger, Emerson Lacey, and Ben Webster for their advice, assistance, and friendship throughout my graduate career. I also want to thank members of Dr. Jocelyn Millar's lab, Jan Bello, Max Collignon, and Steve McElfresh for letting me move in from time to time to use their facilities.

I am grateful to the James San Jacinto Mountain Reserve directors, Becca Fenwick and Jennifer Gee, for facilitating collection of beetles and cooperation in securing the test plots

necessary for my field bioassays. I am also indebted to Peter Morain for his assistance in producing the diagrams of my unique olfactometer designs.

This research was supported in part by the UCR's Dissertation Year Fellowship, the Robert and Peggy van den Bosch Scholarship, a Temecula Valley Wine Society grant, and the UCR's Chancellor's Distinguished Fellowship award.

Chapter two of this dissertation is reproduced with permission from the following published manuscript:

Wheeler, C. A. and Cardé, R. T. 2013. Defensive allomones function as aggregation pheromones in diapausing ladybird beetles, *Hippodamia convergens. Journal of Chemical Ecology*. 39:723-732.

RT Cardé directed and supervised the research, which serves as the basis for this dissertation. Dr. Jocelyn Millar provided technical expertise and analytical equipment.

For my wife, Kimmy

ABSTRACT OF THE DISSERTATION

Odor-Mediated Aggregations of the Aposematic Coccinellid Beetle, *Hippodamia convergens:* Supplementary Functions in Chemical Communication

by

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Doctor of Philosophy, Graduate Program in Evolution, Ecology and Organismal Biology
University of California, Riverside, December 2013
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Preexisting benefits to the signaler and receiver of a candidate semiochemical, facilitates the coevolution necessary to transition that chemical cue into an evolved signal. This natural selection framework would operate within a species in the evolution of multi-functional pheromones, and between species in forms of inter-species communication such as aposematism. The evolution of aposematism depends on a strong coupling between an indicator trait and the defensive trait, yet there is little empirical evidence that supports this relationship. Complex signal theory, also lacking empirical support, predicts specific correlations in the investments among the different multi-modal aposematic displays and the toxicity they advertise.

This dissertation identifies the aggregation pheromones of an aposematic coccinellid, *Hippodamia convergens*, and highlights their supplemental functions, as well as variation in their production and the behavioral responses they evoke. Such an approach is rarely used, but it can

enhance our understanding of the evolution of aggregations, aposematism, and communication in general.

Through laboratory and field assays, I demonstrate that a *H. convergens* aposematic odor (2-isobutyl-3-methoxypyrazine) also functions as an aggregation pheromone among diapausing beetles. In conjunction with this volatile warning signal, *H. convergens* also orient to a cuticular hydrocarbon (tricosane) left behind by walking and aggregating conspecifics. These two aggregation signals interact to mediate the formation and persistence of aggregations at specific hibernacula. Additionally, both have supplementary functions that would transfer additional benefits to aggregating individuals, offsetting the costs of producing them as aggregation pheromones alone: 2-isobutyl-3-methoxypyrazine as an aposematic odor, and tricosane as a component of oviposition deterrent and species-specific signals.

I also quantified the visual and olfactory aposematic displays of *H. convergens* (via spectral analysis of elytra and gas chromatography of whole-body extracts respectively), and demonstrate that they both vary predictably with defensive alkaloids (hippodamine and convergine). Overall, the production of methoxypyrazine aposematic odor is negatively correlated with the degree of alkaloid toxicity. Methoxypyrazines alone do not honestly signal the extent of an individual's toxicity. It is through their synergistic interaction with the honest signal of color, that they exhibit a more nuanced relationship and together function as multi-modal aposematic signals.

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Chapter 1 -

Introduction

The convergent lady beetle, *Hippodamia convergens* (Guérin-Méneville, 1842), is a gregarious, re-migratory, Nearctic species in the family Coccinellidae. There are 60 genera and some 450 species of Coccinellidae native to North America, from nearly 6,000 species worldwide, and of those, roughly 18 belong to the genus *Hippodamia*. *Hippodamia convergens* is commonly used as a biological control agent for aphid and mite pests on cultivated crops. *Hippodamia convergens* spend most of the spring and early summer (March-June) feeding on aphids in mountain valleys, lowland meadows, and agricultural fields throughout North and Central America. Feeding on live prey stimulates full ovarian development in females, and eggs are laid (Rankin and Rankin, 1980b). *Hippodamia convergens* seasonally migrate during autumn and amass by the thousands within the same sheltered microsites as previous generations of migrants, although these small habitat patches are devoid of food resources and isolated within an apparently unsuitable matrix (Hagen, 1962; Hodek and Honêk, 1996; Rankin and Rankin, 1980a). The fall migration of *H. convergens* to specific, revisited mountain hibernacula and the subsequent mating competition that ensues all create conditions of great selective pressure.

Fall migration

The Coccinellidae family of beetles is associated with their main prey, the short-lived, "ephemeral" aphid. Along with migration patterns, a facultative diapause has been selected for which allows coccinellids to synchronize their own life cycles with those of their prey (Hagen, 1962). Coccinellids reproduce when the prey is increasing and become quiescent during prey

shortage. Before man introduced crops to the valleys of California, aphid abundance was very low in the dry summer months, and *H. convergens* expressed summer hibernation called aestivation (Hagen, 1962). In some species, this period of diapause has become obligatory, and aestivation occurs regardless of the summer's high aphid abundance (as a result of irrigation and farmed crops; Hodek, 1967). The traditional *H. convergens* were univoltine (one generation per year or lifetime), but current environmental conditions allow *H. convergens* which do not express an obligatory diapause to be multivoltinism (Hodek and Honěk, 1996).

Newly emerging adults experience a brief period of time (2-3 days) that favors a facultative migration associated with food abundance regardless of the photoperiod. If aphids are abundant, for example due to heavy rain and cultivated crops, the newly emerged females will produce a second generation and most likely remain in foraging habitats for the course of their life (one year). Once the reproductive physiology of female *H. convergens* has been stimulated, the tendency for migration is reduced and females produce eggs rapidly and then die. If aphids are scarce, the period selectively favoring migration will be greatly lengthened in newly emerging beetles and a larger portion of the population will leave (Rankin and Rankin, 1980a). In contrast to females, the summer and fall migration tendencies of males are based solely on food scarcity rather than reproductive status. If the summer exodus does not occur, it is presumed that the short, fall photoperiods will eventually induce some individuals (males, pre-reproductive females [possibly the 2nd or 3rd generations produced]) in the population to migrate (Rankin and Rankin, 1980a). During this migration to overwintering sites, females' reproductive functions are suppressed. The antagonistic relationship between flight and

reproduction is controlled by the hormonal system and is called flight-oogenesis syndrome (Rankin and Rankin, 1980b).

Hippodamia convergens are an example of a re-migrator, meaning that flight leads masses of individuals "from one field of operation to another with the eventual return to the original field of operation by the same individuals" (Hagen, 1962). This is true even in the absence of the ability to direct flight orientation, because individuals respond to particular environmental conditions that favor horizontal movement to and from mountain tops and valleys. Migration occurs at temperatures above 17° C, with undirected upward flight assisted by convection currents. Flight is arrested at 11° C. Individuals can fall as much as 305 meters before a warmer temperature zone of 15° C is reached and flight resumes. During these vertical oscillations, individuals are also blown by horizontal winds. Individuals travel this way over the land until flight is arrested when the temperature ceiling meets mountain slopes. In the example of *H. convergens* migrating from the valleys of California's Sierra Nevada mountains, prevailing westerly winds associated with the pressure system blow beetles toward the mountains. For example, the prevailing winds over Texas are such that they blow migrating beetles from foraging grounds toward overwintering aggregations in the west Texas mountains (Hagen, 1962).

Overwintering behavior

The beetles climatically unsuitable periods or periods of low food abundance in non-feeding developmental stages, which decrease the risk of mortality. The social hibernacula formed during periods of diapause are called aggregations, defined as nonrandom distributions of individuals (Hodek and Honěk, 1996). Aggregating individuals are tightly packed together with

many layers clinging to each other, with the elytra oriented out (Copp 1983; Wheeler personal observations). Overwintering aggregations of *H. convergens* can consist of 10^6 contiguous individuals (Baust and Morrissay, 1975). In one particular case, 2.3 cubic meters of beetles (estimated net total of 4,200,000 individuals) were collected from a single clearing filled with many noncontiguous aggregations (Hagen, 1962).

During the fall at these mountain habitats, individuals feed on non-insect foods (e.g. pollen, nectar) and develop a fatty layer in preparation for hibernation (Hagen, 1962). Initially there is movement around the overwintering aggregation sites, but as temperatures drop all activity ceases and individuals descend into the same protected hibernacula microsites once frequented by previous generations of conspecific migrants (Hagen, 1962; Hodek and Honêk, 1996; Rankin and Rankin, 1980a; Nalepa et al., 2005).

Arrival at these specific, revisited dormancy sites is believed to depend on some combination of hypsotactic orientation (movement toward prominent, isolated objects on the horizon) to montane macrosites, and the possible influence of positive geotaxis, thigmotaxis, hygrotaxis, and negative phototaxis to facilitate the formation of aggregations in microsites with favorable conditions (Hodek and Honêk, 1996; Nalepa et al., 2005). Additionally, aggregations are often located near the temperature flight ceiling. Low temperatures likely induce photonegativity and *H. convergens* descend into the crevasses in and between logs and rocks or beneath the leaf litter. The overwintering aggregations are usually located near mountain streams where the soil retains moisture (Hagen, 1962; Hodek, 1967). The hibernacula move vertically within the column of leaf litter until an optimal 20% moisture content is achieved. The moisture may be important for maintaining a temperature lag between the external

environment and the hibernacula, thus decreasing the effects of sudden cooling. Tests in Minnesota (Hodson, 1937), found that after a steep drop in temperature, it was often 6-10° C warmer in *H. convergens* hibernacula. Moisture is also important because *H. convergens* have been found to imbibe water during dormancy, thus decreasing the risk of desiccation, the most important factor in the mortality of overwintering *H. convergens* (Hodek and Honěk, 1996). Even in situations with high humidity and low temperature, *H. convergens* can suffer extreme water loss that nears fatal levels (Hodson, 1937). It is thus crucial that optimal hibernacula are found.

On warm (>10° C), snow-free days throughout the overwintering period, beetles may become active and exhibit what has been called "thermokinetic behavior" (Benton and Crump, 1979), congregating on sun-exposed surfaces off the ground (Copp, 1983; Honêk et al. 2007). A residual group of inactive beetles remain in the overwintering aggregation, and may serve as a "seed" which assists in the reformation of the overwintering aggregation when temperatures drop after nightfall (Carnes, 1912; Copp, 1983; Yakhontov, 1962). Temperature-regulated phototaxis and aggregation localization (Copp, 1983) are most likely responsible for the emergence and orientation of overwintering *H. convergens* to vertically-prominent, insolated objects. Honêk et al. (2007) described similar daily movements among *Coccinella septempunctata*, whose thermoregulatory behavior induced the beetles to climb onto insolated portions of host plants. Several other researchers also cite similar behaviors with *C. septempunctata* and *Coleomegilla maculate*; they suggest correlations between daily fluctuations in dispersal with temperature, humidity, light, and diel periodicity of searching behavior (Benton and Crump, 1981; Zotov, 1983; Nakamuta, 1987).

It is possible that *H. convergens* aggregations are able to reform year after year and night after night in these same locations due to only the unique conditions of moisture, lighting, and altitude that these optimal hibernacula provide (Hagen, 1962; Nalepa et al., 2005). Additionally, it has been hypothesized, but not adequately tested, that the permanence of these hibernacula is due both to the ability of *H. convergens* to orient to headspace odors of conspecifics, and possibly pheromone deposition from past generations of hibernating conspecifics (Hagen, 1962; Copp, 1983; Majerus, 1997).

Spring dispersal and mating

The dispersal of Coccinellidae from their overwintering sites is a gradual process, generally induced after a prolonged increase of ambient temperature over 10° C during the final stage of winter dormancy (usually in February and March in Southern California; Hodek and Honêk, 1996). Beetles disperse from their aggregation sites and mating ensues before any opportunity to feed. For this reason, the females are mated but reproductively immature, and so migration is not inhibited. Females store the sperm and eventually migrate back to lowland foraging grounds, where growing aphid densities induce ovarian development and oviposition (Rankin and Rankin, 1980a). Little is known about the behaviors and mechanisms of the predispersal mating. There is a passing observation by Savoiskaya (1983) on several species of coccinellids in the arid regions of Kazakhstan forming separate “mating aggregations.”

Whereas the late summer migration (long photoperiod) was much more facultative, depending mostly on food abundance, the spring migration (short photoperiod) is strongly favored regardless of food abundance (Rankin and Rankin, 1980ab). Spring migration begins with upward flight carrying individuals out of the wind shadow created by the high mountain

canyons. In the Sierra Nevada mountains, prevailing northeasterly winds at the temperature flight ceiling blow the beetles away from the mountains. As the temperature decreases towards nightfall, the temperature flight ceiling drops and eventually arrests flight at ground level (Hagen, 1962). Smaller migrations may continue to occur, interrupted with periods of feeding, until ovarian development is complete in the migrant females (Hodek and Honêk, 1996)

The post-diapause flight is strongly favored because it delivers individuals to the optimal environment when their reproductive value is greatest. A reproductive value is a measure of sensitivity to natural selection defined by Slobodkin (1962) as, "the diminution of future population increase produced by removing a single animal of a given age from a population." Natural selection exerts its greatest influence on individuals at a point in their life history when they can make their largest contribution to future generations. Under this premise, migration to an aphid-rich oviposition site is subject to strong favorable selection. Natural selection for summer migration to mountain aggregation sites is much weaker because it is more valuable to maintain actively reproducing populations in aphid-infested areas. The hazards associated with the overwintering diapause periods are great, and thus migration to these aggregation sites is based on prey abundance, and only involves pre-reproductive females (which have enough reproductive value to warrant such risks). Thus, pre-reproductive females emerging in an environment with low aphid abundance will migrate in order to survive this suboptimal environment, while simultaneously placing themselves in close proximity to the other sex. There, it is likely that the migrant females will be mated and may return to the lower elevation feeding sites when aphids are abundant (Rankin and Rankin, 1980b; Hagen, 1962).

Like several other coccinellids, *H. convergens,* is a classic aposematic insect engaging in complex anti-predatory displays. Many coccinellids are distasteful, and advertise this unprofitability with traditional warning coloration (red, yellow, orange/black), methoxypyrazine odor, conspicuousness (including aggregative behavior), and reflex bleeding (reflexive fluid contains concentrated alkaloid toxins and additional pyrazines; Marples et al. 1994, Hodek and Honěk 1996, King and Meinwald 1996). Amplification of coccinellids' visual and olfactory aposematic signal may have been an evolutionary catalyst for the formation of coccinellid aggregations, (Hagen, 1962; Majerus, 1994), but coccinellids tend to aggregate based on temperature and humidity, and not the presence of predators (Benton and Crump, 1979; Copp, 1983). Other fitness advantages of hibernating in groups include the amelioration of harsh environmental conditions by the creation of a microhabitat due to the structure of the aggregation itself, but no direct evidence supports this idea (Hodson, 1937). Interestingly, the transmission of fungal and bacterial pathogens can be much greater within aggregations of overwintering coccinellids than among those coccinellids in solitary behavioral states (Honêk et al., 2007; Nalepa and Weir, 2007). It is therefore the accepted theory, as is the case with other insect groups (Wickman and Rutowski, 1999), that the overwintering aggregations of gregarious coccinellids primarily serve to increase the reproductive efficiency of once highly dispersed populations (Hagen, 1962; Rankin and Rankin, 1980a; Copp, 1983). However, because these aggregations are experiencing imaginal diapause, mating does not occur until they gradually disperse at the end of the winter diapause (Hagen, 1962; Hodek and Honêk, 1996; Rankin and Rankin, 1980a).

The high density of potential mates gradually emerging and becoming active for a time immediately prior to remigration to oviposition sites suggests that these overwintering aggregation sites might serve as forums for mate choice (Arnaud et al., 2003). Because migration has been shown to be a selective mechanism in some species of coccinellids by which the fittest beetles survive to the next season (Honêk et al., 2007), the formation of distinct mating clusters may be another selective mechanism by which the choosy sex can assess a pool of potential mates of a higher relative fitness than those remaining in the quiescent aggregation below.

The seasonal migration of *H. convergens* to revisited overwintering aggregation sites creates a predictable overlap of what might be interpreted as the beetles' home ranges. When such overlap in an organism's home range is predictable in time and space (as with *H. convergens*), clumped mating or leks tend to evolve (Wickman and Rutowski, 1999). The clumped nature of mating at the termination of winter diapause shares many of the same qualities that characterize the mating clusters of other insects as leks: male-biased, non-resource based mate acquisition, and no male parental care (Wickman and Rutowski, 1999).

Though not yet demonstrated in *H. convergens*, several other coccinellids show some degree of female choice (Majerus et al., 1982; Obata, 1988; Nalepa and Weir, 2007). For example, the preference of female *A. bipunctata* for highly melanized males is under genetic control (Majerus et al., 1982). In similar studies with *Harmonia axyridis*, preference for color morphs shifted over the season, with females exerting the most control over sexual selection (Osawa and Nishida, 1992). The mechanism for this choice is unknown, but visual signals are deemed less likely than olfactory signals (Hodek and Ceryngier, 2000). Unfavorable food

conditions cause *H. axyridis* females to reject males' copulation attempts (Obata, 1988). Obata (1988) suggested that *H. axyridis* practice "convenience polyandry" based on the cost of energy expended by females in accepting copulation or resisting males' attempts. Obata (1988) suggested that young females, though more likely to accept copulation than older females, may possess inadequate concentrations of a necessary sex pheromone. No form of cryptic female choice has yet been discovered (Arnaud et al., 2003), but females can store sperm, and sperm competition may exist in some species (Hodek and Ceryngier, 2000). It may be beneficial for females to mate with highly protected males, as there is evidence that male toxins contribute to the chemical defenses of females' eggs in at least one species of coccinellid (Camarano et al. 2006).

Chemical Communication

Hippodamia convergens, and several other species of Coccinellidae, contain distasteful, toxic alkaloids (hippodamine and convergine; King and Meinwald, 1996). These alkaloids are commonly synthesized *de novo* (Rothschild et al., 1970), but some coccinellids sequester them from prey and plants (Durieux, et al., 2010). To repel potential predators many coccinellids can expel droplets of hemolymph tainted with these alkaloids from pores in their femorotibial joint; this is known as reflex-bleeding (Tursch et al., 1974). An aposematic odor, consisting of a blend of three methoxypyrazines (2-isopropyl-3-, 2-secbutyl-, and 2-isobutyl-3-methoxypyrazine) accompanies the alkaloids expelled in reflex fluid (Marples et al. 1994; Hodek and Honěk 1996). These methoxypyrazine allomones are also generally found in the headspace of at least three species of coccinellids (*H. convergens, Harmonia axyridis*, and *Coccinella septumpunctata*;

Cudjoe et al., 2005), and are proven to potentiate predator avoidance learning (Moore et al. 1990).

A majority of the sensory sensilla of *H. convergens* are located on the terminal segments of their antennae (Hamilton et al., 1999). Of these, trichoid sensilla are the most abundant morphological class, and have been suggested to function in long-range chemoreception (Jourdan et al., 1995; Hamilton et al., 1999). Several behavioral studies have shown that many species of Coccinellidae use olfaction in prey location (Zhu et al., 1999; Durieux et al., 2010), oviposition (Seagraves, 2009; Durieux et al., 2010), and species recognition (Hemptinne and Dixon, 2000).

Chemical signals responsible for the mutual attraction of overwintering coccinellid adults are currently being discovered (Durieux et al., 2012; Durieux et al. 2013; Susset et al. 2013; Wheeler and Cardé, 2013). Aggregation pheromones have long been implicated as a possible contributing factor to the formation of coccinellid overwintering aggregations (Carnes, 1912; Edwards, 1957; Yakhontov, 1962; Copp, 1983; Savoiskaya, 1983; Majerus M. E., 1994; Al Abassi et al., 1998; Brown et al., 2006). The same species-specific blends of hydrocarbon semiochemicals have been identified on the surface of coccinellid eggs (Omkar et al., 2004), in the tracks of larvae and adults (Seagraves, 2009), and on the surface of elytra (Hemptinne et al., 1998). This parsimonious versatility of coccinellids' cuticular hydrocarbons, in which serve the multiple purposes of species recognition, habitat assessment, and protection of eggs (Hemptinne and Dixon 2000), have led some to suggest that these compounds may also function as an aggregative pheromone, and persistent markers of hibernaculum sites (Majerus, 1994; Durieux et al., 2010). The saturated, long-chain hydrocarbons found on coccinellid cuticles

are resistant to degradation and will spread easily on hydrophobic plant cuticles (Hemptinne and Dixon, 2000). Such chemical stability and large signal area would facilitate their use as a persistent aggregation pheromone.

Some experimental laboratory bioassays have also suggested that 2-isopropyl-3-methoxypyrazine, one of the aromatic pyrazines found in the headspace of *C. septempunctata*, may act as an attractant (see appendix 1.1 for critique of the methods, results, and conclusions of Al Abassi et al. [1998]). Aggregation sites retain their peculiar odor long after spring migration has left only the dead remains of unsuccessful hibernators (Carnes, 1912; Yakhontova, 1962). It is possible that this defensive methoxypyrazine allomone could have evolved an attractant/aggregation function, as has occurred among other gregarious insects (Blum, 1996). Some argue that if aggregation pheromones exist among the Coccinellidae, they are either short-range/contact pheromones (Nalepa et al., 2000) or they play a secondary role to the influence of visual stimuli, light, and especially humidity, on orientation (Hagen, 1962; Hodek and Honêk, 1996; Nalepa et al., 2005).

I believe that the ability of migrants to find inconspicuous overwintering aggregation sites, the traditional usage of these microsites (Hagen, 1962; Hodek and Honěk, 1996; Rankin and Rankin, 1980a; Nalepa et al. 2005), and the reorganization of smaller satellite aggregations into one primary aggregation during early diapause (Benton and Crump, 1979; Honěk et al. 2007), suggest that there may be some level of chemically-mediated orientation involved. Chemical information conveyed using pheromones is a common mode of communication involved in "social" aggregations that form due to individuals' direct responses to each other (especially among arthropods; Wertheim et al. 2005). *Hippodamia convergens* present an ideal

system with which to study chemical signaling. Their status as gregarious aposematic insects provides the opportunity to apply concepts of semiochamical parsimony and complex signal theory to further our knowledge and understanding of the evolution of aposematism and aggregations.

Literature Cited

Al Abassi, S., Birkett, M. A., Pettersson, J., Pickett, J. A., and Woodcock, C. M. 1998. Ladybird beetle odor identified and found to be responsible for attraction between adults. *Cellular and Molecular Life Sciences.* 54:876-879.

Al Abassi, S., Birkett, M. A., Pettersson, J., Pickett, J. A., Wadhams, L. J., and Woodcock, C. M. 2001. Response of the ladybird parasitoid *Dinocampus coccinellae* to toxic alkaloids from the seven-spot ladybird, *Coccinella septempuntata*. *Journal of Chemical Ecology*. 27:33-43.

Agarwala, B.K., and Yasuda, H. 2001. Overlapping oviposition and chemical defense of eggs in two co-occurring species of ladybird predators of aphids. *Journal of Ethology*. 19:47-53.

Arnaud, L., Spinneux, Y., and Haubruge, E. 2003. Preliminary observations of sperm storage in *Adalia bipunctata*: sperm size and number. *Applied Entomology and Zoology*. 38: 301-304.

Baust, J. G., and Morrissey, R. E. 1975. Supercooling phenomenon and water-content independence in overwintering beetle, *Coleomegilla maculata*. *Journal of Insect Physiology*. 21:1751-1754.

Benton, A. H., and Crump, A. J. 1979. Observations on aggregation and overwintering in the Coccinellid beetle *Coleomegilla maculata* (Degeer). *Journal of the New York Entomological Society.* 87:154-159.

Blum, M. S. 1996. Semiochemical parsimony in the Arthropoda. *Annual Review of Entomology.* 41:353-374.

Brown, A. E., Riddick, E. W., Aldrich, J. R., and Holmes, W. E. 2006. Identification of (-)-beta-caryophyllene as a gender-specific terpene produced by the multicolored Asian lady beetle. *Journal of Chemical Ecology.* 32:2489-2499.

Carnes, E. 1912. An explanation of the hibernating habits of *Hippodamia convergens*. *Monthly Bulletin of the California State Commission of Horticulture.* 1:177-188.

Copp, N. H. 1983. Temperature-dependent behaviours and cluster formation by aggregating ladybird beetles. *Animal Behaviour.* 31:424-430.

Cudjoe, E., Wiederkehr, T. B., and Brindle, I. D. 2005. Headspace gas chromatography-mass spectrometry: a fast approach to the identification and determination of 2-alkyl-3-methoxypyrazine pheromones in ladybugs. *Analyst* 130:152-155.

Durieux, D., Verheggen, F. J, Vandereycken, A., Joie, E., and Haubruge, E. 2010. Syntheses bibliographique: l' ecologie chimique des coccinelles. *Biotechnology, Agronomy, Society and Environment*. 14:17-20.

Durieux, D., Fischer, C., Brostaux, Y., Sloggett, J. J., Deneubourg, J-L., Vandereycken, A., Joie, E., Wathelet, J-P., Lognay, G., Haubruge, E., and Verheggen, F. J. 2012. Role of long-chain hydrocarbons in the aggregation behaviour of *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellida). *Journal of Insect Physiology.* 58:801-807.

Durieux, D., Fassotte, B., Vanderplank, M., Deneubourg, J-L., Fischer, C., Lognay, G., Haubruge, E., and Verheggen, F. J. 2013. Substrate marking by an invasive ladybeetle: Seasonal changes in hydrocarbon composition and behavioural responses. *PLoS One.* 8:e61124.

Edwards, J. G. 1957. Entomology above timberline: the attraction of ladybird beetles to mountain tops. *Coleoptera Bulletin.* 11:41-46.

Hagen, K. S. 1962. Biology and ecology of predaceous Coccinellidae. *Annual Review of Entomology* 7:289-326.

Hamilton, R. M., Dogan, E. B., Schaalje, G. B., and Booth, G. M. 1999. Olfactory response of the lady beetle *Hippodamia convergens* (Coleoptera: Coccinellidae) to prey related odors, including a scanning electron microscopy study of the antennal sensilla. *Environmental Entomology*. 28:812-822.

Hemptinne J.-L., and Dixon A. F. 2000. Defense, oviposition and sex: semiochemical parsimony in two species of ladybird beetles (Coleoptera, Coccinellidae)? A Short Review. *European Journal of Entomology*. 97:443-447.

Hemptinne, J. L., Lognay G., and Dixon A. F. G. 1998. Mate recognition in the two-spot ladybird beetle, *Adalia bipunctata*: role of chemical and behavioural cues. *Journal of Insect Physiology*. 44:1163-1171.

Hemptinne, J. L., Lognay G., M. Doumbia, and A. F. G. Dixon. 2001. Chemical nature and persistence of the oviposition deterring pheromone in the tracks of the larvae of the two spot ladybird, *Adalia bipunctata* (Coleoptera: Coccinellidae). *Chemoecology.* 11:5.

Hemptinne, J. L., G. Lognay, C. Gauthier, and A. F. G. Dixon. 2000. Role of surface chemical signals in egg cannibalism and intraguild predation in ladybirds (Coleoptera : Coccinellidae). *Chemoecology*. 10:123-128.

Honêk, A., Martinkova, Z., and Pekar, S. 2007. Aggregation characteristics of three species of Coccinellidae (Coleoptera) at hibernation sites. *European Journal of Entomology*. 104:51-56.

Hodek, I. and Ceryngier, P. 2000. Sexual activity in Coccinellidae (Coleoptera): a review. *European Journal of Entomology.* 97:8.

Hodek, I. and Honěk, A. 1996. Ecology of Coccinellidae. Kluwer Academic Publishers, Boston.

Hodson, A. C. 1937. Some aspects of the role of water in insect hibernation. *Ecological Monographs*. 7:271-315.

Jourdan, H., Barbier, R., Bernard, J., and Ferran, A. 1995. Antennal sensilla and sexual dimorphism of the adult ladybird beetle *Semiadalia undecimnotata* (Schn. Coleoptera: Coccinellidae*). International Journal of Insect Morphology and Embryology*. 24: 307-322.

King, A. G. and Meinwald, J. 1996. Review of the defensive chemistry of coccinellids. *Chemical Reviews.* 96:1105-1122.

Majerus, M. E. N. 1994. Ladybirds. Harper Collins, London.

Majerus, M. E. N. 1997. How is *Adalia bipunctata* (Linn.) (Coleoptera: Coccinellidae) attracted to overwintering sites? *Entomologist* 116:212-217.

Majerus, M. E. N., Odonald, P., and Weir, J. 1982. Female mating preference is genetic. *Nature*. 300:521-523.

Moore, B. P., Brown, V. W., and Rothschild, M. 1990. Methylalkylpyrazines in aposematic insects, their hostplants and mimics. *Chemoecology* 1:43-51.

Marples, M. E. N., Vanveelen, W., and Brakefield, P. M. 1994. The relative importance of colour, taste and smell in the protection of an aposematic insect *Coccinella septempunctata*. *Animal Behaviour.* 48:967-974.

Nakashima, Y., Birkett, M. A., Pye, B. J., Pickett, J. A., and Powell, W. 2004. The role of semiochemicals in the avoidance of the seven-spot ladybird, C*occinella septempunctata*, by the aphid parasitoid, *Aphidius ervi*. *Journal of Chemical Ecology.* 30:1103-1116.

Nakamuta, K. 1987. Diel rhythmicity of prey-search activity and its predominance over starvation in the ladybeetle, *Coccinellaseptempunctata*. *Physiological Entomology*. 12:91-98.

Nalepa, C. A., Kennedy, G. G., and Brownie, C. 2005. Role of visual contrast in the alighting behavior of *Harmonia axyridis* (Coleoptera: Coccinellidae) at overwintering sites. *Environmental Entomology.* 34:425-431.

Nalepa, C. A., Kidd, K. A., and Hopkins, D. I. 2000. The multicolored Asian lady beetle (Coleoptera : Coccinellidae): orientation to aggregation sites. *Journal of Entomological Science*. 35:150-157.

Nalepa, C. A., and Weir, A. 2007. Infection of Harmonia axyridis (Coleoptera: coccinellidae) by *Hesperomyces virescens* (Ascomycetes: Labooulbeniales): Role of mating status and aggregation behaviour. *Journal of Invertebrate Pathology*. 94:196-203.

Obata, S. 1988. Mating refusal and its significance in females of the ladybird beetle, *Harmonia axyridis*. *Physiological Entomology.* 13:193-199.

Omkar, A. Pervez, and A. K. Gupta. 2004. Role of surface chemicals in egg cannibalism and intraguild predation by neonates of two aphidophagous ladybirds, *Propylea dissecta* and *Coccinella transversalis*. *Journal of Applied Entomology*. 128:5-691-695.

Osawa, N., and Nishida, T. 1992. Seasonal variation in elytral color polymorphism in *Harmonia axyridis* (the ladybird beetle): the role of nonrandom mating. *Heredity*. 69:297-307.

Rankin, M. A., and Rankin, S. 1980a. Some factors affecting presumed migratory flight activity of the convergent ladybeetle, *Hippodamia-convergens* (Coccinellidae, Coleoptera). *Biological Bulletin*. 158:356-369.

Rankin, S. M., and Rankin, M. A. 1980b. The hormonal-control of migratory fight behaviour in the convergent ladybird beetle, *Hippodamia convergens*. *Physiological Entomology*. 5:175-182.

Rothschild M., von Euw J., and Reichstein T. 1970. Cardiac glycosides in the oleander aphid. *Journal of Insect Physiology*. 16:1141-1145.

Savoiskaya, G. I. 1983. Kokcinellidy. (In Russian) Alma-Ata, Kazachskoi, USSR.

Seagraves, M. P. 2009. Lady beetle oviposition behavior in response to the trophic environment. *Biological Control*. 51:313-322.

Slobodkin, L. B. 1962. Energy in animal ecology. *Advances in Ecological Research*. 69:1017-1048.

Susset, E. C., Ramon-Portugal, F., Hemptinne, J.-L., Dewhirst, S. Y., Birkett, M. A., and Magro, A. 2013. The role of semiochemicas in short-range location of aggregation sites in *Adalia bipunctata* (Coleoptera, Coccinellidae). *Journal of Chemical Ecology.* 39:592-601.

Takizawa, T., Yasuda, H., and Agarwala, B. K. 2000. Effect of three species of predatory ladybirds on oviposition of aphid parasitoids. *Entomological Sciences*. 3:465-469.

Tursch, B., Daloze, D., Braekman, J. C., Hootele, C., Cravador, A., Losman, D., and Karlsson, R. 1974. Chemical ecology of arthropods 9: structure and absolute configuration of hippodamine and convergine, 2 novel alkaloids from American ladybug *Hippodamia convergens* (Coleoptera-Coccinellidae). *Tetrahedron Letters.* 15:409-412.

Verheggen, F. J., Fagel, Q., Heuskin, S., Lognay, G., Francis, F., and Haubruge, E. 2007. Electrophysiological and behavioral responses of the multicolored Asian lady beetle, *Harmonia axyridis* Pallas, to sesquiterpenes semiochemicals. *Journal of Chemical Ecology*. 33:2148-2155.

Wertheim, B., van Baalen, E.-J. A., Dicke, M., and Vet, L. E. 2005. Pheromone-Mediated Aggregation in Nonsocial Arthropods: An Evolutionary Ecological Perspective. *Annual Review of Entomology.* 50:321-346.

Wickman, P.O., and Rutowski, R. L. (1999). The evolution of mating dispersion in insects. *Oikos*. 84, 463-472.

Yakhontov, V. 1962. Seasonal migrations of lady-birds *Brumus octosignatus* (Gebl) and *Semiadalia undecimnotata* (Schneid) in Central Asia. *Proceedings of the 11th International Congress of Entomology*. 11:21-23.

Zhu, J., Cosse, A. A., Obrycki, J. J., Boo, K. S., and Baker, T. C. 1999. Olfactory reactions of the twelve-spotted lady beetle, *Coleomegilla maculata* and the green lacewing, *Chrysopera carnea* to semiochemicals released from their prey and host plant: electroantennogram and behavioral responses. *Journal of Chemical Ecology.* 25:1163-1177.

Zotov, V. A. 1983. Exogenous and endogenous components of the diel rhythm of activity in *Coccinella-septempunctata* (Coleoptera, Coccinellidae). *Zoologichesky Zhurnal*: 62, 1654-1662.

Appendices

Appendix 1.1 Critique of Al Abassi et al. (1998)

Al Abassi et al. (1998) used coupled gas chromatography-organoleptic evaluation to identify 2-isopropyl-3-methoxypyrazine as the compound responsible for the characteristic odor of *C. septempunctata* adults. This compound was then tested for an aggregative function in a 2-arm olfactometer. Results led the authors to believe that this compound induced an attraction-arrestment response. Air was introduced into the arena from two adjacent arms (the other two arms and half of the central arena were blocked off); clean air issued from the control arm, and different formulations of odorant issued from the treatment arm. The different treatments consisted of a single adult *C. septempunctata*, the vacuum distillate equivalent to 0.04 of an adult, and a synthetic source of 2-isopropyl-3-methoxypyrazine. The position of a single adult within the arena was noted every 2 minutes for 20 minutes (i.e. scan sampling), and after 5-10 replications, the locational frequency data was analyzed. Beetles were located significantly more often in the treatment arm than in the control arm, with the synthetic compound yielding the greatest differential (Al Abassi et al., 1998). This experiment and its analysis of scan-sampled data are not wholly diagnostic (analysis did not take into account repeated measures, or low replication), and cannot conclusively identify attraction and arrestment, or their underlying navigational mechanisms. Such location frequency distribution analysis should be coupled with additional analysis at a finer temporal scale, along with actual locomotory data (e.g. Ponsonby and Copland, 1995; Hamilton et al., 1999).

Literature Only Cited in Appendix 1.1

Ponsonby, D. J. and Copland, M. J. W. 1995. Olfactory responses by the scale insect predator *Chilocorus nigritus* (F.) (Coleoptera: Coccinellidae). *Biocontrol Science and Technology*. 5:83-93.

Chapter 2 -

Defensive allomones function as aggregation pheromones in diapausing *Hippodamia convergens*

Abstract

Identification of the stimuli responsible for the formation of an aggregation can be used to distinguish between social and non-social aggregations, and help in the process of identifying the adaptive benefits of the gregarious behavior. The convergent lady beetle (*Hippodamia convergens)* forms dense aggregations during winter diapause. The mechanisms of conspecific attraction and hibernacula site selection of *H. convergens* are not well understood. We evaluated the role of three defensive compounds in the formation of *H. convergens* aggregations in laboratory and field bioassays. Diapausing *H. convergens* aggregated within the section of an arena exposed to methoxypyrazines. 2-Isobutyl-3-methoxypyrazine (IBMP) caused the strongest aggregative effect. Beetles also aggregated to some doses of 2-sec-butyl-methoxypyrazine (SBMP), but appeared to be repelled at higher doses. A third constituent, 2-isopropyl-3-methoxypyrazine (IPMP), generally had little effect on the distribution of beetles, although the highest dose tested was repellent. Beetles also aggregated to a blend of these methoxypyrazines in their natural ratio. During fall migration to overwintering sites, significantly more beetles aggregated in artificial hibernacula baited with IBMP, confirming its function as an aggregation pheromone. These three pyrazines also function as warning odors that in conjunction with other aposematic displays (contrasting red and black coloration, gregarious

behavior, reflex bleeding) contribute to the multi-modal, anti-predator defence of coccinellid beetles and some other arthropods. Confirmation of the role of some methoxypyrazines in coccinellid aggregations suggests that these defensive allomones have been co-opted for intraspecific communication.

Introduction

Although the benefits of aggregations are well known (e.g., Gamberale and Tullberg, 1998; Parrish and Edelstein-Keshet, 1999), less attention has been paid to the proximal causes facilitating their formation and maintenance (Muller, 1998; Sempo et al., 2009). Aggregations often are described as being the result of adaptive social behaviors, such as inter-individual attraction, when in reality they may have formed incidentally due to the random movements of individuals (Parrish and Edelstein-Keshet, 1999), or by active movements of individuals in response to patchy environmental stimuli, rather than in response to conspecific signals (Majerus, 1997; Stephens and Sutherland, 1999). There is uncertainty as to the behavioral mechanisms responsible for aggregations in the convergent lady beetle (*Hippodamia convergens*). This beetle spends the spring and summer months as a relatively solitary predator of aphids, and migrates in the early fall to adjacent mountains where it forms massive overwintering aggregations at the bases of trees and on rocks, fallen logs, or patches of duff. A single aggregation can consist of a couple of thousand to millions of conspecific individuals tightly packed on top of one another (Majerus, 1997; Hodek et al., 2012).

Although the seasonal occurrence of these massive aggregations is well known, the stimuli responsible for the attraction of overwintering adults to these traditional sites are not fully understood. Abiotic stimuli may have a strong influence over coccinellids' mass arrival at specific dormancy sites that are revisited yearly (Benton and Crump, 1979; Nalepa et al., 2005; Honěk et al., 2007; Hodek et al., 2012). Orientation responses to vertically contrasting physical features (hypsotaxis), humidity, temperature, light levels, and physical contact (thigmotaxis), could result in the incidental accumulation of many individuals at the few microsites possessing

optimal environmental conditions. This “shelter seeking” behavior suggests that the aggregations themselves may have no evolutionary function and are only a result of habitat limitation. Numerous observations note, however, that coccinellids assemble into aggregations at only a small percentage of the seemingly optimal microhabitats available in their local environment (Benton and Crump, 1979; Honěk et al., 2007).

Gregarious behavior at traditional sites suggests that a social factor, such as attraction to conspecifics, may be involved (Grether and Donaldson, 2007). Aggregation pheromones have long been suspected as a contributing factor in the intrinsic aggregation tendency and strong site fidelity of *Hippodamia convergens* (Carnes, 1912; Hawkes, 1926; Copp, 1983), and several other gregarious coccinellids (Weiss, 1913; Edwards, 1957; Yakhontov, 1962; Majerus, 1994; Brown et al., 2006). Information conveyed using pheromones is a common mode of communication mediating “social” aggregations that form due to individuals’ responses to each other (especially among arthropods, Wertheim et al., 2005).

There is evidence that the coccinellid aggregation signal may be in part non-volatile, suggesting that there is indirect conspecific attraction via site-marking. Majerus (1997) found that fewer *A. bipunctata* migrants aggregated at overwintering microsites that had been thoroughly cleansed of any residue potentially left behind by the previous season’s overwintering conspecifics. Frass was eliminated as a sole cue, and Majerus reasoned that a persistent marking pheromone might be responsible for the aggregation of new migrants at these traditional hibernacula. Hydrocarbons deposited on glass and metal surfaces by walking and aggregating *Harmonia axyridis* have recently been shown to elicit an aggregative response in conspecifics (Durieux et al., 2012).

While these hydrocarbon sources of aggregation signals are likely perceived only by direct contact with the hibernacula substrate (Durieux et al., 2012), volatile components may be responsible for directly attracting migrants to overwintering conspecifics over longer distances. Copp (1983) found that *H. convergens* aggregated in areas of an arena exposed to headspace odor from conspecifics. Similarly, conspecific headspace odor, and specifically the 2-isopropyl-3-methoxypyrazine (IPMP) constituent of that odor, influenced the distribution of *Coccinella septempunctata* assayed individually in a 2-choice olfactometer (Al Abassi et al., 1998). Although aggregative behavior was not directly assayed, Al Abassi et al. (1998) proposed that the IPMP warning odor may potentially serve as an aggregation pheromone component in *C. septempunctata*. IPMP is one of three methoxypyrazine allomones (the others being: 2-sec-butyl- [SBMP] and 2-isobutyl-3-methoxypyrazines [IBMP]) whose presence was also confirmed by Cudjoe et al. (2005) in the gregarious coccinellid species, *H. axyridis* and *H. convergens* (quantities per beetle in the latter ≈ 5.7 µg IPMP, 0.35 µg SBMP, and 1.08 µg IBMP per beetle). *Hippodamia convergens*, a beetle known for its gregarious behavior, contains a higher concentration of each of these methoxypyrazines, with three times more IPMP than *H. axyridis* and more than 200 times that of *C. septempunctata* (Cudjoe et al., 2005).

Besides being passively released into the headspace of live coccinellids, these three methoxypyrazines along with toxic alkaloids (hippodamine and convergine in *H. convergens*, King and Meinwald, 1996) are secreted from the femorotibial joint of "perturbed" coccinellids (Marples et al., 1994; Hodek et al., 2012). Such reflex bleeding is a defensive behavior, and the accompanying methoxypyrazines function as an aposematic odor to potentiate predators' learned avoidance response to these toxic beetles (Moore et al., 1990). Like several other

coccinellids, *H. convergens* is a classic aposematic insect engaging in complex anti-predatory displays. Their distastefulness is advertised not only with their reflex bleeding and methoxypyrazine odors, but also with traditional warning coloration (red/yellow/orange and black patterns) and conspicuousness (Dolenská et al., 2009 [this includes aggregative behavior, Ruxton and Sherratt, 2006]).

We hypothesized that volatile odors released by diapausing *H. convergens* account, at least in part, for the aggregation of conspecifics at particular overwintering microsites. Specifically, we determined whether methoxypyrazine allomones of *H. convergens* also function as an aggregation pheromone. The localized release of a volatile aggregation pheromone within an olfactometer is expected to induce aggregative behavior within that specific section of the arena. Those compounds that induce localized gregarious behavior under laboratory conditions are also expected to induce similar responses under field conditions during the arrival of fall migrants to overwintering macrosites. Identification of a volatile aggregation pheromone would help explain how naïve, migrating *H. convergens* are able to orient to the few isolated overwintering aggregations of conspecifics that dot an expansive montane landscape.

Methods and Materials

Experimental Animals.

Adult *H. convergens* were collected from the University of California's James San Jacinto Mountain Reserve, near Idyllwild, CA. Collections were made in late October from a series of overwintering aggregations found along the banks of a seasonal stream (lat: 33.811063, lon: -116.774204, elev. 1,650 m). Beetles collected from this site were considered to be in diapause. The sex-ratio of aggregating *H. convergens* was assumed to be ≈50:50 (Hagen, 1962), and a

sample of beetles from one aggregation was used to check this assumption. The sample consisted of 465 males and 466 females, confirming that overwintering aggregations contain an even sex ratio. Beetles were sexed by morphological features of their terminal sternites (Majerus and Kearns, 1989).

All other samples of beetles were housed in a single plastic, rectangular container (91 x 20 x 12 cm) containing a bed of ≈5 x 42 cm crumpled paper strips (Kimwipes™, Kimberly-Clark Professional, Roswell GA.) and two moistened cotton dental wicks. This container was held in an environmental chamber programmed to mimic conditions typical of overwintering hibernacula (10L:14D, conditions cycled daily in a fixed pattern within a range of 5-13° C and 65-90% RH). Such environmental conditions maintain *H. convergens* in a state of diapause (Bennett and Lee, 1989). Overwintering conditions were based on two years of recordings from weather stations near an overwintering aggregation at Mount San Antonio, Los Angeles County, CA (lat: 34.24495, lon: -117.65875, elev. 1,650 m), archived records retrieved from the Mount Baldy Fire Department (elev. 1291 m; Asst. Chief G. Hendrickson personal communication, April 15 2011), and from the Western Regional Climate Center's data (elev. 1495 m) on the James San Jacinto Mountains Reserve website.

Arena Aggregation Bioassay.

The laboratory bioassay was based on Copp (1983), but with alterations to the arena (Fig. 1) and experimental protocols. The diameter of the circular arena was doubled (from ≈10 to 20 cm), and it was constructed from aluminum instead of plastic. The interior surfaces of the arena walls were painted with fluon (Insect-a-Slip™, BioQuip Products Inc., Rancho Dominquez, CA), a substance which makes surfaces too slippery for beetles to walk upon. An exhaust system suspended 2 cm above the arena walls vented treatment odor from the open arena and from

the bioassay room in general. Between each trial, arenas were disassembled, washed in soapy warm water (Sparkleen™, Fisherbrand, Pittsburgh PA.), rinsed with tap water followed by ethyl alcohol, and baked at 135° C for 24 hours. The Kimwipe™ paper floor was replaced for each trial.

Treatment odors were placed in a randomly selected compartment before the floor was installed. For the *in vivo* headspace treatment, 50 *H. convergens* were placed in a randomly selected compartment. Beetles were prevented from climbing the walls of their compartment and the underside of the arena floor by painting all the inner surfaces of the aluminum end cap with fluon. Results from blank control trials were used to test for any inherit directional bias of the experimental arena. *In vivo* headspace trials then tested the hypothesis that volatiles in the headspace of aggregating *H. convergens* are at least partly responsible for the localized formation of conspecific aggregations (Copp, 1983).

Grey rubber septa (1-F SS 1888 GRY, West Co., Lionville PA.) were used to release methoxypyrazine odor treatments. The three methoxypyrazines [2-isobutyl- (IBMP); 2-sec-butyl-, (SBMP); and 2-isopropyl-3-methoxypyrazines, (IPMP); Aldrich Chemical Co., Milwaukee, WI] were diluted in methylene chloride. Solutions were formulated so that four different doses (5.5, 20, 55, and 200 ng) of each methoxypyrazine could be tested individually by applying a standard volume of 50 µl of solution to the septum well. The order of methoxypyrazines and doses tested were randomized. Odor treatments also included a 1:1:1 blend of the three methoxypyrazines, and a blend mimicking the ratio of methoxypyrazines quantified from the headspace of recently killed *H. convergens* (4:1:16; Cudjoe et al., 2005). Individual methoxypyrazine treatments were tested first, so that an appropriate dose could be selected for testing these blends. The 1:1:1

blend contained 20 ng each of the methoxypyrazines and the 4:1:16 natural ratio blend contained 8, 2 and 32 ng, respectively. These amounts were based on doses of the three methoxypyrazines that individually evoked aggregation and did not cause repellency (see Results). For each trial, one treated septum was prepared in addition to three control septa charged with 50 µl of methylene chloride. Septa were mounted on a hole-punched paper stand and given 10 min for evaporation of solvent before assembling the rest of the arena.

The rotational heading of the arenas' compartments were randomized between trials. Two arenas were run simultaneously under separate exhaust hoods, with 25 diapausing mixed sex beetles introduced into each. The bioassay room was maintained in uninterrupted darkness at 11° C ± 0.5 for 24 hours. Most if not all beetles would arrest along (if not touching) the walls of the arena after this time period. The arena floor was then photographed from a fixed position. From the photographs, each beetle's location was recorded as the category of quadrant it was located above (treatment or one of three blank quadrants). Each beetle's relative distance from the treatment section was also measured in bearing degrees (with the 0° mark set to the middle of the treatment quadrant).

Field Aggregation Bioassay.

Field tests of the purported aggregation pheromones were performed within two traditional *H. convergens* overwintering macrosites at the James San Jacinto Mountain Reserve. The two overwintering sites were located approximately 1.5 km apart and straddled the same mountain stream (Indian Creek) along stretches flowing in a southwest direction. Both aggregation macrosites were immediately adjacent to level clearings where no beetles had been observed aggregating during the past two winter seasons (Wheeler, personal observation): site 1 on the southeast bank (lat: 33.811063, lon: -116.774204, elev. 1,650 m), site 2 on the northwest bank

(lat: 33.815296, lon: -116.769135, elev. 1,682 m). During early summer (March 2012), when the habitat was devoid of gregarious *H. convergens*, both clearings were raked free of duff in order to remove any persistent odor cues. A grid of 9 cement tiles (30.5 x 30.5 x 1.5 cm, grey) spaced 4 m apart was then installed at each of these sites. The columns of the grid were arrayed in parallel to the stream. Each tile was buried into the ground so that the top face of the tile was made flush with the soil surface. Steel legs, 1 cm in length, held another cement tile directly over the partially-buried tile like a roof.

After initial installation of the artificial hibernacula, beetle counts were routinely conducted at each untreated hibernaculum to ensure that no beetles aggregated. The field was regularly visited until flying migrants appeared on 11th August 2012. On this date, odor-treated rubber septa were placed in the middle of each partially buried cement tile. A multiple Latin square design was used to distribute the three odor treatments across all 18 artificial hibernacula. Both sites held three hibernacula replicates of each odor treatment. Every two days, the number of beetles at each hibernaculum was counted and treatments were refreshed until a total of five sets of recordings were made (totaling to 30 replicates for each treatment). Results from the arena bioassay informed the doses to be used in field experiments. Three treatments were selected for the field trials: a non-odor blank, a blend containing 20 ng of each of the three methoxypyrazines, and IPMP alone at a dose of 20 ng.

Statistical Analysis

The distribution of individuals in the laboratory bioassay was characterized by analyzing their angular degree position using circular statistical software (Oriana v3.21, Kovach Computing Services, Pentraeth, Wales). These unidirectional angular data were converted to bidirectional axial data (0-360° to 0-180^0 by pooling together positions to the left and right of the treatment

zone's center axis). This conversion step removes the potential of bi-modal angular data from producing deceptive mean vectors (e.g., aggregations where none exist) or false conclusions of uniform distribution.

Data from all replicates of a treatment were pooled for analysis. Data were handled in this way because *H. convergens* are highly gregarious and thus degree positions within each trial were expected to be clumped. Pooling these individually-clumped distributions together and calculating the mean vector (μ), reveals whether or not there is a directional bias as to where individual beetles arrest across replicates of the same treatment. A mean vector angle within a range of $315° \leq \mu \leq 45°$ (or $0° \leq \mu \leq 22.5°$ after axial conversion), indicates a directional bias for the treatment quadrant. The mean vector length (*r*) indicates the strength of this directional bias. A mean vector length of 1 equals a high concentration of points and 0 signifies an even dispersal of points. The uniform distribution of the beetles' positions between each trial was tested with a *Rayleigh test*. A *V-Test* was also run using an expected mean that fell within the treatment quadrant (standardized as 0°). A significant result from a *V-Test* indicates that the distribution of beetles deviates significantly from uniformity, and that that non-uniformity is biased towards the treatment quadrant.

Data from the field experiment were analyzed using GLM with repeated measures (SAS v9.1.3, SAS Institute INC., NC, USA). If necessary, this SAS procedure could also control for any significant effects due to field site location, count date, and hibernaculum position within the grid array. The data were also tested for normal distribution and equal variance. There was significantly unequal variance in the data (*Levene's Test*: $F_{90} = 4.77$, $P < 0.01$; *Bartlett's Test*: $X^2_2 = 44.6617$, $P < 0.0001$), and thus the data were $\sqrt{(x + 1 \ / \ 2)}$ transformed before analysis.

Results

The uniform distribution of beetles in the control bioassay confirmed that there was no directional bias inherent in the arena design (Table 1). Although beetles tended to clump together into one or two aggregations within each control treatment trial, over all trials the positions of these aggregations appeared to be random. In contrast, the frequency of beetles recorded above the in vivo headspace treatments, was two times greater than the frequency expected if there had been no directional bias (Table 1). Angular degree data from in vivo odor treatments departed significantly from uniform distribution. The mean vector fell within the treatment zone, and had a substantially larger r than the control treatment (Fig. 2). A V-Test confirmed a significant preference for the center of the in vivo conspecific odor treatment zone (Table 1).The three different synthetic methoxypyrazine treatments were tested at three or four different doses in laboratory bioassays (Table 1). Each methoxypyrazine could not be tested at all four doses during the time window that the overwintering season allowed. Beetles showed significant preference for all doses of IBMP (5.5, 20, 55 ng; Table 1). All doses of SBMP (5.5, 20, and 55 ng) caused a non-uniform distribution of beetles, but only with the 20 ng dose was the distribution significantly biased towards the treatment quadrant (Table 1). The 5.5 ng SBMP dose yielded a mean vector 11° outside of the treatment zone, and had a V-Test critical factor of 0.002 (a value above our 0.001 significance threshold; Fig. 3). The 55 ng dose yielded a significant directional bias in the opposite direction of the treatment quadrant (Table 1). Directional bias appeared to be inconclusive for IPMP treatments. Aggregations departed significantly from uniform distribution for the 5.5 and 55 ng IPMP doses, but with a directional bias deviating approximately 90° away from the center of the treatment quadrant (Fig. 3). The highest dose of 200 ng (tested only with IPMP in this experiment) had a directional bias nearly

opposite to the treatment zone, suggesting a repellent effect similar to the 55 ng SBMP dose. The significant preference for the treatment odor was maintained in both the 1:1:1 and 4:1:16 blend treatments that were tested. (Fig. 4, Table 1).

Migrating H. convergens were observed entering and arresting in the artificial hibernacula that had been installed in overwintering macrosites. Migrants also were observed aggregating on nearby, natural hibernacula that had been utilized in past seasons. The different odor treatments used in the artificial hibernacula had a significant effect on the number of beetles that aggregated within them (GLM: $F_{90} = 117.21$, $P < 0.001$). Significantly more beetles aggregated in the hibernaculum treated with either methoxypyrazine treatment than in the non-odor (blank) hibernaculum (Fig. 5). No significant effect of site location, date, or position within the grid arrangement of hibernacula was evident.

Discussion

The experiments reported here provide evidence that the defensive allomones of *H. convergens* have been co-opted as an aggregation pheromone. Most beetles were arrested in aggregations that formed within the section of the arena exposed to odors of a hidden aggregation of live conspecifics. This result suggested that *H. convergens* exhibit an aggregative preference for some volatile chemical constituent(s) of conspecific headspace odor. Further trials with odor-treated septa confirmed an aggregative function of two common methoxypyrazine constituents of coccinellid headspace odor, IBMP in particular, as well as SBMP. Artificial hibernacula baited with a methoxypyrazine blend or with IBMP alone also initiated aggregation during early fall migration.

Organisms searching for optimal habitat are expected to be attracted to conspecifics if either their presence at a site serves as an indirect cue of habitat quality (conspecific cueing, Muller, 1998; Vet, 1999) or if the newcomer will benefit from the presence of those conspecifics themselves (Allee effect, Stephens and Sutherland, 1999). In the case of *H. convergens* and other coccinellids, however, migrants are observed to be initially dispersed among all the nearby seemingly optimal habitat before eventually assembling into larger aggregations at only a fraction of those hibernacula locally available (Benton and Crump, 1979; Honěk et al., 2007; Wheeler, personal observation). This particular preference for shared hibernacula, as opposed to adjacent empty ones that had been previously inhabited, indicates that a pheromone alone does not necessarily serve as a signal for habitat quality or habitat detection, but that the presence of conspecifics themselves may increase the fitness of diapausing coccinellids at these social hibernacula.

Social aggregations among animals serve a variety of functions, including protection from predators, creation of an optimal microclimate, accelerating/synchronizing ontological development, increasing reproductive efficiency (Danks, 2002), and overcoming host/prey defenses (Raffa et al, 1993). Coccinellid aggregations consist of diapausing, non-feeding adults, and evidence for the creation of a microclimate is ambiguous (Benton and Crump, 1979; Danks, 2002). It therefore remains that coccinellid aggregation may have an anti-predator or reproductive function, or both. Gregariousness is a common behavioral trait of aposematic insects similar to *H. convergens* (Ruxton and Sherratt, 2006), and the fact that *H. convergens* aggregation pheromone components are also part of their defensive allomone suggests that the resulting aggregation itself might have an anti-predator function as well.

Aggregation pheromone signaling in *H. convergens* (and potentially the closely-related aggregating coccinellids, *H. axyridis* and *C. septempunctata*) may have evolved from its well-established role as a warning odor in antipredator defense. Because aggregations have been shown to strengthen the warning signal of color (Gamberale and Tullberg, 1998; Riipi et al., 2001), they may also strengthen the warning signal of odor by potentiating the detection, discrimination, and avoidance learning of predators (inter-signal interaction hypotheses of complex signal theory, Hebets and Papaj, 2005). The benefits of anti-predatory defense would defray the cost of producing the methoxypyrazines as an aggregation pheromone alone (Blum, 1996; Skelhorn and Ruxton, 2008), and conceivably individuals could gain further anti-predator defense benefits from the cumulative contribution of warning odor emitted from the other members of the aggregation (Kaufmann, 1966; Aldrich and Blum, 1978; Moore et al., 1990). Thus the individuals would benefit from the multifunctional methoxypyrazine odor surrounding an aggregation as the odor would serve both as a chemical signal to find the aggregation, and once there, as a communal defense against potential predators.

Although pheromone-mediated aggregations are widespread in insects (Wertheim et al., 2005), there are relatively few cases of a species' defensive allomones also serving as aggregation pheromones. Exceptions are the tenebrionid beetles, *Blaps sulcata* (Kaufmann, 1966), *B. mucronata* (Tannert and Hien, 1973), and several species of the subtribe Stizophina (Geiselhardt et al., 2009). There are some examples, however, of defensive secretions or glands also serving a sexual function (in the families of Miridae, Romaleidae, Staphylinidae, Formicidae [Blum, 1996], and Pyrochroinae [Eisner et al. 1996]). The three *H. convergens* methoxypyrazines, and pyrazines in general, are common warning odors in aposematic insects and plants (Moore et

al., 1990). The apparent convergent evolution of pyrazines as a significant agent of Müllerian mimicry may be due to their pungent odors, low olfactory threshold, stable chemical nature, straight-forward biosynthesis, and abundance in nature (Woolfson and Rothschild, 1990). These are beneficial characteristics that might have also facilitated the evolution of their parsimonious roles as alarm pheromones (several ponerine and formicine ants, Brown and Moore, 1979), trail-laying pheromones (a species of *Atta* and various myrmecine leaf-cutter ants, Cross et al., 1979), male sex pheromones (a tephritid fruit fly, Baker et al., 1982), and attractive marking pheromones (a few philanthine and nyssonine wasps, Borg-Karlson and Tengö, 1980).

Al Abassi et al. (1998) demonstrated that individual *C. septempunctata* spent more time within the section of an olfactometer that was exposed to the headspace odors of either a conspecific or IPMP. This was determined by noting the position of an individual test beetle within a two-armed olfactometer every 2 minutes for 20 minutes. This method cannot establish the locomotory maneuvers responsible, as either an increase in turning angles/frequency (klinokinesis), a decrease in locomotion (negative orthokinesis or arrestment), or movement upwind might be potential mechanisms. Their results, however, are consistent with behaviors that may be responsible for the formation of aggregations. Our bioassay experiments examined a different species with similar odor treatments, and monitored gregarious behavior. Similarly, our results indicate headspace odor affects the distribution of beetles, but instead of IPMP, IBMP was identified as the behaviorally significant constituent. Although our experiments do not establish the exact locomotory maneuvers responsible, they confirm that the end result is the formation of aggregations. The term, “aggregation pheromone” is a teleological classification, because it does not reflect the locomotory mechanisms responsible for the formation of the

aggregation (Kennedy, 1978). Further work should examine whether the aggregations that form due to these methoxypyrazine signals are a result of chemically-mediated arrestment or locomotory kinesis/taxis. The cuticular hydrocarbons left behind by previous aggregating individuals (Durieux et al., 2012), may induce a different set of locomotory responses that interact with responses to the volatile methoxypyrazines to complete the aggregation signal.

Hippodamia convergens and several other coccinellids are important biological control agents for aphid and mite pests on cultivated crops. These highly mobile predators have broad habitat ranges and are able to persist in a spatially and temporally variable agricultural landscape (Duelli et al., 1990). Most native species (especially *H. convergens*) are extremely sensitive to declines in prey abundance and health (Evans, 2004). Populations readily disperse from agricultural fields and may not return early enough to control subsequent pest outbreaks, thus compromising their efficiency as natural control agents (Hagen, 1962; Rankin and Rankin, 1980). This situation is likely exacerbated by the competitive exclusion of native coccinellids from the agricultural habitats they once dominated by two introduced coccinellids, *H. axyridis* and *C. septempunctata* (Evans, 2004). The methoxypyrazine aggregation pheromones identified here might be capable of keeping dispersive coccinellids within agricultural fields or adjacent refugia, thus providing more reliable pest control. Conversely, lures could be developed which might divert coccinellids away from vineyards. Recently, coccinellids have been incorporated inadvertently into grape harvests, contaminating wine with their potent defensive compounds (Pickering et al., 2004).

Literature Cited

Al Abassi, S., Birkett, M. A., Pettersson, J., Pickett, J. A., and Woodcock, C. M. 1998. Ladybird beetle odor identified and found to be responsible for attraction between adults. *Cellular and Molecular Life Sciences.* 54:876-879.

Aldrich, J. R. and Blum, M. S. 1978. Aposematic aggregation of a bug (Hemiptera: Coreidae): defensive display and formation of aggregations. *Biotropica.* 10:58-61.

Baker, R., Herbert, R. H., and Lomer, R. A. 1982. Chemical components of the rectal gland secretions of male *Dacus cucurbitae*, the melon fly. *Experientia.* 38:232-233.

Bennett, L. E. and Lee, R. E. 1989. Simulated winter to summer transition in diapausing adults of the lady beetle (*Hippodamia convergens*). *Physiological Entomology.* 14:361-367.

Benton, A. H. and Crump, A. J. 1979. Observations on aggregation and overwintering in the coccinellid beetle *Coleomegilla maculata* (DeGeer). *Journal of the New York Entomological Society.* 87:154-159.

Blum, M. S. 1996. Semiochemical parsimony in the Arthropoda. *Annual Review of Entomology.* 41:353-374.

Borg-Karlson, A-K. and Tengö, J. 1980. Pyrazines as marking volatiles in Philanthine and Nyssonine wasps (Hymenoptera: Sphecidae). *Journal of Chemical Ecology.* 6:827-835.

Brown, W. V. and Moore, B. P. 1979. Volatile secretory products of an Australian Formicine ant of the genus *Calomyrmex* (Hymenoptera: Formicidae). *Insect Biochemistry.* 9:451-460.

Brown, A. E., Riddick, E. W., Aldrich, J. R., and Holmes, W. E. 2006. Identification of (-)-beta-caryophyllene as a gender-specific terpene produced by the multicolored Asian lady beetle. *Journal of Chemical Ecology.* 32:2489-2499.

Carnes, E. 1912. An explanation of the hibernating habits of *Hippodamia convergens*. *Monthly Bulletin of the California State Commission of Horticulture.* 1:177-188.

Copp, N. H. 1983. Temperature-dependent behaviours and cluster formation by aggregating ladybird beetles. *Animal Behaviour.* 31:424-430.

Cross, J. H., Byler, R. C., Ravid, U., Silverstein, R. M., Robinson, S. W., Baker, P. M., Sabinodeoliveira, J., Jutsum, A. R., and Cherrett, J. M. 1979. The major component of the trail pheromone of the leaf-cutting ant, *Atta sexdens rubropilosa* Forel, 3-Ethyl-2,5-Dimethylpyrazine. *Journal of Chemical Ecology.* 5:187-203.

Cudjoe, E., Wiederkehr, T. B., and Brindle, I. D. 2005. Headspace gas chromatography-mass spectrometry: a fast approach to the identification and determination of 2-alkyl-3-methoxypyrazine pheromones in ladybugs. *Analyst.* 130:152-155.

Danks, H. V. 2002. Modification of adverse conditions by insects. *Oikos.* 99:10-24.

Dolenská, M., Nedvěd, O., Veselý, P., Tesařová, M., and Fuchs, R. 2009. What constitutes optical warning signals of ladybirds (Coleoptera: Coccinellidae) towards bird predators: colour, pattern or general look? *Biological Journal of the Linnean Society*. 98:234-242.

Duelli, P., Studer, M., Marchand, I., and Jakob, S. 1990. Population movements of arthropods between natural and cultivated areas. *Biological Conservation.* 54:193-207.

Durieux, D., Fischer, C., Brostaux, Y., Sloggett, J. J., Deneubourg, J-L., Vandereycken, A., Joie, E., Wathelet, J-P., Lognay, G., Haubruge, E., and Verheggen, F. J. 2012. Role of long-chain hydrocarbons in the aggregation behaviour of *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellida). *Journal of Insect Physiology.* 58:801-807.

Edwards, J. G. 1957. Entomology above timberline: the attraction of ladybird beetles to mountain tops. *Coleoptera Bulletin.* 11:41-46.

Eisner, T., Smedley, S. R., Young, D. K., Eisner, M., Roach, B. and Meinwald, J. 1996. Chemical basis of courtship in a beetle (Neopyrochroa flabellate): Cantharidin as precopulatory "enticing" agent. *Proceedings of the National Academy of Sciences.* 93:6494-6498.

Evans, E.W. 2004. Habitat displacement of North American ladybirds by an introduced species. *Ecology.* 85:637-647.

Gamberale, G. and Tullberg, B. S. 1998. Aposematism and gregariousness: the combined effect of group size and colouration on signal repellence. *Proceedings of the Royal Society of London B Biological Sciences.* 265:889-894.

Geiselhardt, S., Schmitt, T., and Peschke, K. 2009. Chemical composition and pheromonal function of the defensive secretions in the subtribe Stizopina (Coleptera, Tenebrionidae, Opatrini). *Chemoecology.* 19:1-6.

Grether, G. F. and Donaldson, Z. R. 2007. Communal roost site selection in a Neotropical Harvestman: habitat limitation vs. tradition. *Ethology.* 113:290-300.

Hagen, K. S. 1962. Biology and ecology of predaceous Coccinellidae. *Annual Review of Entomology.* 7:289-326.

Hawkes, O. A. M. 1926. On the massing of the ladybird, *Hippodamia convergens* (Coleoptera), in the Yosemite Valley. *Proceedings of the Zoological Society of London.* 1926:693-705.

Hebets, E. A. and Papaj, D. R. 2005. Complex signal function: developing a framework of testable hypotheses. *Behavioral Ecology and Sociobiology.* 57:197-214.

Hodek, I., van Emden, H. F., and Honěk, A. 2012. Ecology and Behaviour of the Ladybird Beetles (coccinellidae). Wiley-Blackwell, Oxford.

Honěk, A., Martinkova, Z., and Pekár, S. 2007. Aggregation characteristics of three species of Coccinellidae (Coleoptera) at hibernation sites. *European Journal of Entomology.* 104:51-56.

Kaufmann, T. 1966. Observations on some factors which influence aggregation by *Blaps sulcata* (Coleoptera - Tenebrionidae) in Israel. *Annals of the Entomological Society of America.* 59:660-664.

Kennedy, J. S. 1978. The concepts of olfactory 'arrestment' and 'attraction'. *Physiological Entomology.* 3:91-98.

King, A. G. and Meinwald, J. 1996. Review of the defensive chemistry of coccinellids. *Chemical Reviews.* 96:1105-1122.

Majerus, M. E. N. 1994. Ladybirds. Harper Collins, London.

Majerus, M. E. N. 1997. How is *Adalia bipunctata* (Linn.) (Coleoptera: Coccinellidae) attracted to overwintering sites? *Entomologist.* 116:212-217.

Majerus, M. E. N. and Kearns, P. 1989. Ladybirds: Naturalist Handbooks 10. Richmond Publishing Co Ltd., Slough, England.

Marples, M. E. N., Vanveelen, W., and Brakefield, P. M. 1994. The relative importance of colour, taste and smell in the protection of an aposematic insect *Coccinella septempunctata*. *Animal Behaviour.* 48:967-974.

Moore, B. P., Brown, V. W., and Rothschild, M. 1990. Methylalkylpyrazines in aposematic insects, their hostplants and mimics. *Chemoecology.* 1:43-51.

Muller, K. L. 1998. The role of conspecifics in habitat settlement in a territorial grasshopper. *Animal Behaviour.* 56:479-485.

Nalepa, C. A., Kennedy, G. G., and Brownie, C. 2005. Role of visual contrast in the alighting behavior of *Harmonia axyridis* (Coleoptera: Coccinellidae) at overwintering sites. *Environmental Entomology.* 34:425-431.

Parrish, J. K. and Edelstein-Keshet, L. 1999. Complexity, pattern, and evolutionary trade-offs in animal aggregation. *Science.* 284:99-101.

Pickering, G., Lin, J., Riesen, R., Reynolds, A., Brindle, I., and Soleas, G. 2004. Influence of *Harmonia axyridis* on the sensory properties of white and red wine. *American Journal of Enology and Viticulture*. 55:153-159.

Raffa, K. F., Phillips, T. W., and Salom, S. M. 1993. Strategies and mechanisms of host colonization by bark beetles, pp. 103-128, in T. D. Schowalter and G. M. Filip (eds.). Beetle Pathogen Interactions in Conifer Forests. Academic Press, San Diego, California.

Rankin, M. A., and Rankin, S. 1980. Some factors affecting presumed migratory flight activity of the convergent ladybeetle, *Hippodamia convergens* (Coccinellida, Coleoptera). *Biological Bulletin.* 158:356-369.

Riipi, M., Alatalo, R. V., Lindstrom, L., and Mappes, J. 2001. Multiple benefits of gregariousness cover detectability costs in aposematic aggregations. *Nature.* 413:512-514.

Ruxton, G. D. and Sherratt, T. N. 2006. Aggregation, defense and warning signals: the evolutionary relationship. *Proceedings of the Royal Society B Biological Sciences.* 273:2417-2424.

Sempo, G., Canonge, S., Detrain, C., and Deneubourg, J. L. 2009. Complex dynamics based on a quorum: decision-making process by cockroaches in a patchy environment. *Ethology.* 115:1150-1161.

Skelhorn, J. and Ruxton, G. D. 2008. Ecological factors influencing the evolution of insects' chemical defenses. *Behavioral Ecology.* 19:46-153.

Stephens, P. A. and Sutherland, W. J. 1999. Consequences of the Allee effect for behaviour, ecology and conservation. *Trends in Ecology and Evolution.* 14:401-405.

Tannert, W. and Hien, B. C. 1973. Evidence and function of an assembling scent and an alerting scent of *Blaps mucronata* (Coleoptera Tenebrionidae). *Biologisches Zentralblatt.* 92:601-612.

Vet, L. E. M. 1999. From chemical to population ecology: infochemical use in an evolutionary context. *Journal of Chemical Ecology.* 25:31-49.

Weiss, H. B. 1913. Some Tropic Reactions of *Megilla maculata* (DeG) and notes on the hydrotropism of certain mosquitoes. *Canadian Entomologist.* 45:85-87.

Wertheim, B., van Baalen, E-J. A., Dicke, M., and Vet, L. E. M. 2005. Pheromone-mediated aggregation in nonsocial arthropods: an evolutionary ecological perspective. *Annual Review of Entomology.* 50:321-346.

Woolfson, A. and Rothschild, M. 1990. Speculating about pyrazines. *Proceedings of the Royal Society of London B Biological Sciences*. 242:113-119.

Yakhontov, V. V. 1962. Seasonal migrations of lady-birds *Brumus octosignatus* Gebl. and *Semiadalia undecimnotata* Schneid in Central Asia. *11th International Congress of Entomology Vienna.* 3:21-23.

Tables and Figures

Table 1 Circular statistical analysis of the bidirectional axial position (0°-180°) of *Hippodamia convergens* from alkylmethoxypyrazine treatments in the laboratory bioassay.

alkylmethoxypyrazine	% in trt.	*Rayleigh test*		mean vector	*V-Test*		
amount (ng)	quadrant[a]	Z	*P*[b]	μ	V	*P*	n
Blank control							
0	28	0.34	0.71	n/a	n/a	n/a	120
In vivo beetles							
(50 indv.)	49	50.63	<0.001	16°	0.47	<0.001	225
2-isobutyl-3-							
5.5	69	39.98	<0.001	28°	0.56	<0.001	99
20	63	51.89	<0.001	31°	0.51	<0.001	148
55	64	49.09	<0.001	14°	0.98	<0.001	100
2-sec-butyl-							
5.5	38	12.89	<0.001	56°	0.20	0.002	100
20	52	15.39	<0.001	36°	0.33	<0.001	95
55	27	13.44	<0.001	161°	-0.25	1	195
2-isopropyl-3-							
5.5	6	23.32	<0.001	84°	0.05	0.25	101
20	27	3.06	0.047	n/a	0.09	0.10	100
55	7	31.15	<0.001	105°	-0.10	0.98	197
200	3	18.21	<0.001	144°	-0.35	1	97
Blend							
20:20:20	64	57.74	<0.001	28°	0.52	<0.001	171
8:2:32	48	58.38	<0.001	32°	0.47	<0.001	188

[a] The percent of beetles, pooled across all replicates of each treatment, which were located above the treatment quadrant (standardized to 0^0 to 45^0, with the actual treated lure at 0^0).

[b] A critical value of 0.001 was selected because those samples with a $0.001<p<0.05$ had a dose value and sample size too low to reliably calculate the standard error of the mean

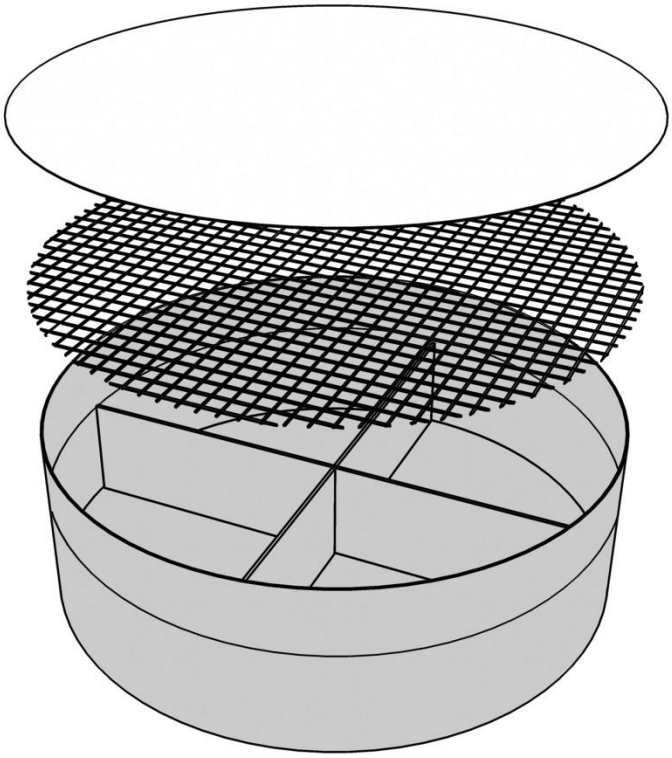

Figure 1 An exploded-view drawing of the experimental olfactometer. Aluminum sheets were riveted into place within an aluminum end cap (20 cm in diameter and 5 cm in depth) to divide the volume into four equal compartments. In each trial, one of these randomly selected compartments served as the odor release point. A metal screen and a 37 by 42 cm Kimwipe™ paper sheet were stretched over the open end of the end cap, flush with the top of the compartment dividers. A 12.7 cm long aluminum coupler was attached to the end cap, thus holding the porous arena floor in place and creating a surrounding wall

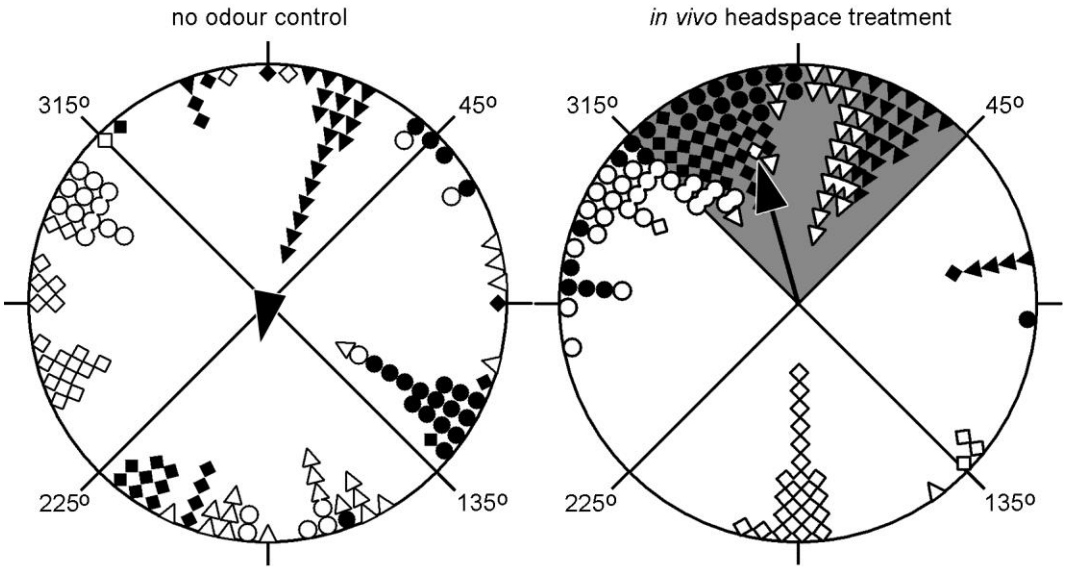

Figure 2 Diagrammatic rose plots displaying the frequency of *Hippodamia convergens* indviduals' positions in angular degrees along the perimeter of a circular still-air olfactometer. Six representative replicate trials are presented in each histogram. Trials are designated by different symbols and shading. Each trial consisted of 25 beetles. The arrow extending from the center of each circle indicates the direction of the mean vector, with its length symbolizing mean vector length. The localized release of this treatment odor is depicted by the shaded quarter

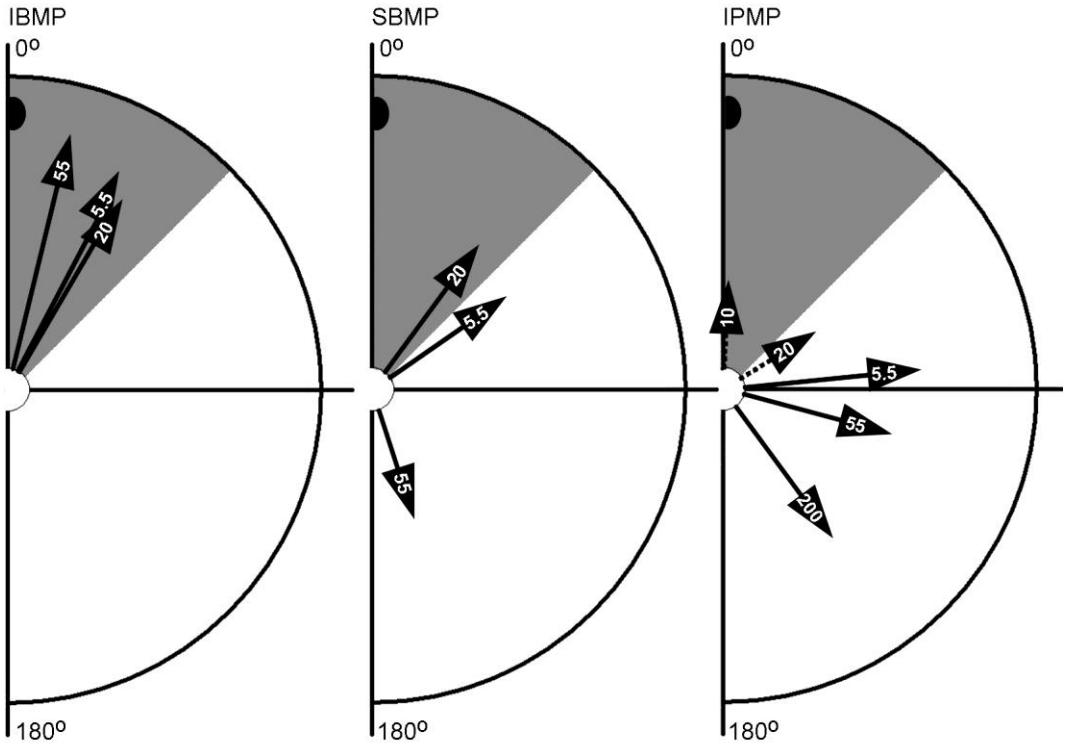

Figure 3 The mean bi-directional location (0°-180°) of *Hippodamia convergens* in relation to the localized release of three different methoxypyrazine compounds over a range of concentrations. The black half-circle at 0^0, represents the odor-baited lure at the center of the treatment quadrant (shaded section). The farthest distance from the treatment is at 180^0. Arrows, labeled with the dosage in ng, point in the direction of the mean vector. An arrow's length represents the strength of the bidirectional bias (mean vector length); the dashed arrow is not significant.

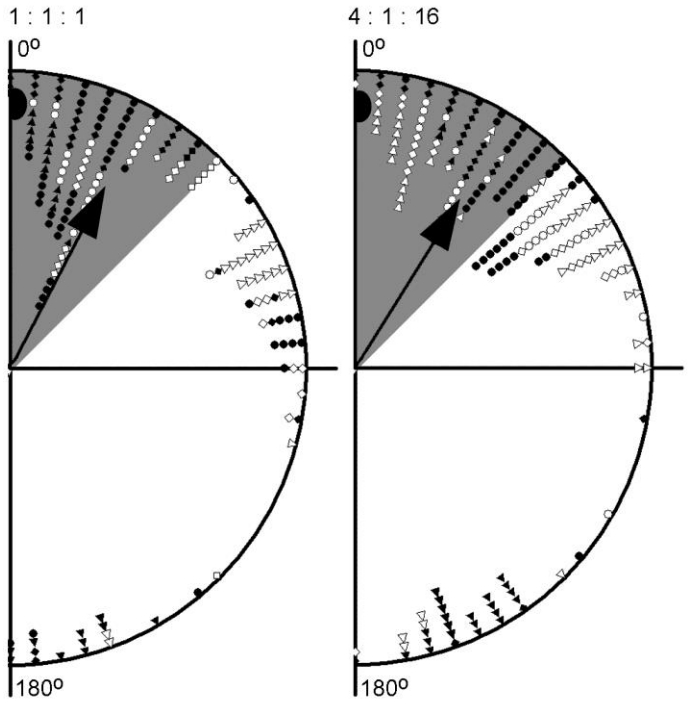

Figure 4 The mean bi-directional location (0°-180°) of *Hippodamia convergens* in relation to the localized release of methoxypyrazine blends in laboratory bioassays. The 1:1:1 treatment consisted of 20 ng of each methoxypyrazine. The 4:1:16 treatment (the natural ratio) consisted of 8 ng of IBMP, 2 ng of SBMP, and 32 ng of IPMP. These pie charts are similar to Fig. 3, but with the addition of filled and hollow symbols depicting each beetle's location from each trial

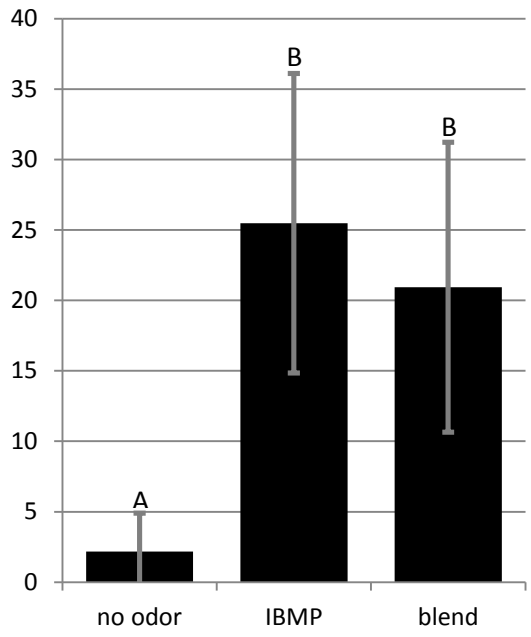

Figure 5 Bar graph depicting the average number of *Hippodamia convergens* beetles counted in an artificial hibernaculum treated with one of three odor treatments. Error bars show standard deviation and letters indicate significant differences between the treatments. The IBMP treatment contained 20 ng of IBMP, and the blend treatment contained 20 ng of each of three methoxypyrazines (IBMP, SBMP, and IPMP). N=30

Chapter 3 -

Following in their footprints: Cuticular hydrocarbons as overwintering aggregation site markers in *Hippodamia convergens*

Abstract

The convergent lady beetle (*Hippodamia convergens*) forms large overwintering aggregations at revisited montane microsites far removed from their summer foraging grounds. Although orientation responses to habitat features can explain the arrival of migrants at the general overwintering macrosite, the role that pheromones play in the accumulation of individuals in inconspicuous hibernacula microsites is not fully understood. Through Y-maze bioassays and gas chromatography and mass spectrometry, we found that *H. convergens* orient towards hydrocarbons previously deposited on their walking surfaces by conspecifics. Tricosane (C_{23}) is primarily responsible for this chemically-mediated orientation. Footprint extracts, as well as C_{23} alone, induce the eventual accumulation in the field of migrant *H. convergens* at artificial hibernacula, confirming their probable role as aggregation signals. Aggregations persisted over many days when footprint extracts were applied in conjunction with the previously identified 2-isobutyl-3-methoxypyrazine aggregation pheromone. The C_{23} hydrocarbon functions as a semiochemical that interacts with responses to methoxypyrazines to effectively mediate formation of persistent aggregations of diapausing conspecifics at specific microsites. Also discussed is the potential effect that C_{23} has as a persistent scent marker in establishing the traditional use of hibernacula.

Introduction

The convergent lady beetle, *Hippodamia convergens*, is an important biological control agent for aphid and mite pests. These highly mobile predators readily disperse from agricultural fields during prey shortages, thus compromising their efficiency as natural control agents (Hagen, 1962; Rankin and Rankin, 1980a). This situation is likely exacerbated by the competitive exclusion of the native *H. convergens* from the agricultural habitats they once dominated by introduced species of ladybeetle (Evans, 2004). Conversely, aggregations of these same ladybeetle species cause problems in vineyards when they are unintentionally incorporated into grape harvests and contaminate wine (Brown et al., 2006). In early fall, *H. convergens* migrate to adjacent mountain ranges where they form aggregations and overwinter in the same sheltered hibernacula used by previous generations of diapausing conspecifics (Copp, 1983; Hodek and Honěk, 1996).

Although the seasonal occurrence of these aggregations is well known, the stimuli responsible for the attraction of beetles to these traditional shelter sites are not fully understood. Wheeler and Cardé (2013) identified volatile compounds in *H. convergens* that functioned as aggregation pheromones in both laboratory and field bioassays. *Hippodamia convergens* primarily aggregated within the section of a bioassay arena exposed to odors from a hidden aggregation. This species, as well as several other species of ladybeetle, emit 3 specific methoxypyrazines (Cudjoe et al., 2005). *Hippodamia convergens* showed a significant preference for forming aggregations in the part of an arena treated with two methoxypyrazines, 2-isobutyl-3-methoxypyrazine and 2-sec-butyl-methoxypyrazine (Wheeler and Cardé, 2013). Beetles also aggregated in response to a natural ratio of these methoxypyrazines. During the fall

migration of *H. convergens* to mountain overwintering sites, artificial hibernacula treated with methoxypyrazines contained significantly larger aggregations than untreated shelters. As fall migration progressed into winter diapause, however, aggregations at these methoxypyrazine-baited artificial aggregations appeared to disband, whereas aggregations at traditionally-used hibernacula in the neighboring area grew (Wheeler, personal observation). It would thus appear that although these methoxypyrazines have some effect on the formation of aggregations, there must be other stimuli involved in their maintenance. One could also argue that these methoxypyrazines could not act alone as an aggregation signal, because hibernacula are reused yearly by naïve migrants (Hagen, 1962; Hodek and Honěk, 1996; Rankin and Rankin, 1980; Nalepa et al., 2005), and the methoxypyrazine signals likely dissipate or degrade over the summer and thus could not serve as a persistent site marker.

There is evidence that the coccinellid aggregation signal may be, in part, non-volatile, thus suggesting that there is indirect conspecific attraction via site marking. Majerus (1997) found that fewer *Adalia bipunctata* migrants aggregated at overwintering microsites that had been thoroughly cleansed of any residue potentially left behind by the previous year's overwintering conspecifics. Majerus (1997) reasoned that a persistent marking pheromone at these traditional hibernacula might be responsible for the aggregation of new immigrants. A blend of saturated and unsaturated hydrocarbons ranging from 23 to 31 carbons in length deposited on glass and metal surfaces by walking and aggregating *Harmonia axyridis* elicited an aggregative response among diapausing conspecifics (Durieux et al., 2012). The cuticular hydrocarbons left behind by previous aggregating individuals may induce a different set of locomotory responses that interact with responses to the volatile methoxypyrazines to

complete the aggregation signal. This hypothesis is supported by recent evidence from a laboratory bioassay in which *Adalia bipunctata* spent more time in the vicinity of a source of methoxypyrazines that also had extracts of conspecific hydrocarbons nearby (Susset et al., 2013).

Cuticular hydrocarbons can also be deposited by the tarsi as an insect walks, forming a chemical trail (Kosaki and Yamaoka, 1996) that affects orientation (Wilms and Eltz, 2008; Steinmetz et al., 2003). Coccinellid "footprints" can deter oviposition and foraging patch selection (reviewed in Seagraves, 2009; in *H. convergens* Michaud and Jyoti, 2007) and can function as allomones when they affect the orientation and foraging search strategies of other species (Pettersson et al., 2005). Similar blends of hydrocarbons are present on the elytra of adults and on the surface of eggs, where they function in species/gender recognition and predation defense respectively (reviewed in Hemptinne and Dixon, 2000) The versatile communicative functions of these hydrocarbons and their persistent nature (Hemptinne et al., 2001) suggest that cuticular hydrocarbons may be good candidates for contact pheromones that constitute a persistent hibernaculum site "label." Persistent aggregation pheromones may act as an external form of social memory, facilitating the year-to-year maintenance of aggregation sites (Boyd and Richerson, 1985; Donaldson and Grether, 2007).

The orientation responses of diapausing *H. convergens* were tested in a Y-maze treated with conspecific footprint compounds. These footprint compounds were analyzed and identified with coupled gas chromatography-mass spectrometry, and compared with extracts from the beetles' cuticle. The behavioral assay was repeated with extract fractions and synthetic blends to identify behaviorally relevant semiochemicals. Field bioassays, consisting of artificial

hibernacula baited with components identified from the extracts, were used to determine whether one or more of these compounds influenced the aggregation responses of overwintering migrant beetles.

Methods and Materials

Experimental Animals

Adult diapausing *H. convergens* were collected from the University of California's James San Jacinto Mountain Reserve, near Idyllwild, CA, USA. Collections were made in September from a series of newly forming overwintering aggregations found along the banks of a seasonal stream (lat: 33.811063, lon: -116.774204, elev. 1,650 m). Beetles were housed in plastic, rectangular containers (91 x 20 x 12 cm), with a bed of ≈5 x 42 cm crumpled paper strips (KimwipesTM, Kimberly-Clark Professional, Roswell GA.) and two moistened cotton dental wicks. Beetles were maintained in a state of diapause (as described in Wheeler and Cardé, 2013), except for one sample of beetles, which were transferred to a greenhouse under diapause-terminating conditions one month prior to the y-maze assay (16L:8D photoperiod, 20-27° C and 68% RH [Rankin and Rankin 1980; Bennett and Lee, 1989]; fed an excess of *Acyrthosiphon pisum* reared on *Vicia faba* [Arnaud et al. 2003]). Diapause was terminated in this greenhouse beetle cohort, as evidenced by vitellogenesis in the female ovarioles, increased sperm count in female bursa copulatrix, and observed oviposition (Appendix 3.1).

Preparation of extracts

Footprint extracts from adults were prepared by placing 24 live beetles in a glass petri dish (92 mm diam.) for 48 hours at environmental conditions typical of their mountain-top overwintering climate (described in Chapter 2). The beetles were removed from the petri dish and all interior surfaces of both lids were scrubbed with two 30 mg glass wool balls soaked in distilled hexane.

The dishes were then rinsed with 4 ml of hexane, and the rinse was eluted through the glass wool. The glass wool was further eluted with an additional 4 ml of clean hexane. The resulting 8 ml of extract was stored in a vial at -25° C. Samples of the extract were fractionated on a silver nitrate-impregnated silica gel column eluted with hexane and 20% cyclohexene in hexane to separate alkanes from alkenes.

Extracts of whole beetles and their legs also were prepared. For the whole body extracts, 16 adult beetles (killed by freezing) were vortexed in 4 ml of hexane for 5 minutes. For the beetle leg extract, all the legs of 16 beetles (previously killed by freezing), were removed and vortexed in 2 mL of hexane for 5 minutes

Coupled Gas Chromatography-Mass Spectrometry (GC-MS)

Hexane extracts were analyzed on a Hewlett Packard 6890 series gas chromatograph (Agilent Technologies: Santa Clara CA.), equipped with a non-polar capillary column (DB-5MS, 30 m x 0.25 mm x 0.25 µm film; J&W Scientific, Folsom CA.), connected to an H-P 5973 series mass selective detector. The GC injection port was set to 260° C and the transfer line to 300° C. The column temperature was held at 60° C for 2 min, then increased to 220° C at 40° C/min, and then to 315° C at 4° C/min. Helium was used as the carrier gas at 1 ml/min, and samples were injected in splitless mode with a purge time of 2 min. Electron impact ionization (70 eV) mass spectra were measured on an H-P 6890 GC interfaced to an H-P 5973 mass selective detector. The GC was equipped with a DB-17 column (30 m x 0.25 mm x 0.25 µm film; J&W Scientific, Folsom CA.). Samples were injected splitless at 280ºC, with a temperature program of 100ºC/1 min, 10ºC/min to 280º, hold for 20 min.

Hydrocarbons were tentatively identified by matches with the NIST 98 mass spectral database. Identifications were confirmed by matching retention indexes and mass spectra with those of standards. Retention indexes were calculated with reference to straight-chain alkanes (chain lengths 22-30, 32, 34). Compounds were quantified with reference to a synthetic internal standard, 5-methyl-heptacosane ($5MeC_{27}$), added at a concentration of 95 ng/µl.

Chemicals

n-Tricosane ($C_{23}H_{48}$) was obtained from Alltech Associated, Inc. (Deerfield IL.), *n*-tetracosane ($C_{24}H_{50}$) from Applied Science laboratories Inc. (State College PA.), *n*-pentacosane ($C_{25}H_{52}$) and *n*-hexacosane ($C_{26}H_{54}$) from the Aldrich Chemical Company (St. Louis MI.), and *n*-heptacosane ($C_{27}H_{56}$) from Fluka Chemia (Burchs, Switzerland). For behavioral studies, individual and mixed solutions of these hydrocarbons were prepared in distilled hexane (Fisher Scientific, Fair Lawn NJ.) at the concentrations at which they were found in the beetle extracts. Grey rubber septa (1-F SS 1888 GRY, West Co., Lionville PA.) were used to release the 2-isobutyl-methoxypyrazine (IBMP; Aldrich Chemical Co., Milwaukee WI.) odor treatments. IBMP was diluted in dichloromethane to a concentration of 0.4 ng/µl.

Y-maze Bioassay

A two-choice Y-maze bioassay was used to evaluate the pheromonal role of compounds found within the footprint extracts of beetles. The bioassay device consisted of strips of white, unruled index cards adhered in the shape of a 'Y' to an inclined (120°) pane of glass (basal trunk, 130 mm x 6 mm; choice arms, 65 mm x 6 mm split by 90°). The pane of glass was coated with Fluon® (BioQuip Products Inc., Rancho Dominquez CA.) to restrict beetle locomotion to the 'Y'. The bioassay was performed in a lit room held at 19° C. Non-directional, uniform light was ensured by covering the arena with a translucent plastic dome traditionally used for studio photography

(Cloud Dome, Inc., Lafayette CO.). Trials were run during the beetles' photophase. Beetles were placed individually in a halved soufflé cup (P100-0100 29.5 mL translucent polystyrene, Solo Cup Company, Lake Forest IL.) creating a floor and wall enclosing the bottom 33 mm of the main trunk of the 'Y'. Beetles were allowed 5 minutes to ascend onto the main trunk of the 'Y'. A choice was recorded if a beetle entered one of the two arms of the 'Y' and stayed on that arm for longer than 2 seconds. Trials were stopped if the beetle remained on the main trunk of the 'Y' for longer than 5 minutes. We also timed latency between the moment the beetle first climbed onto the main trunk of the 'Y' to when it made a first choice. An initial treatment consisted of pre-exposing index cards laid out on the floor of a rectangular container (330 x 190 x 110 mm), to the locomotory and aggregative activity of 125 beetles for 48 hours (approximately equivalent exposure to that of the footprint extract derived from 24 beetles on the petri dish). These cards were then cut into strips to be used as the treatment arm of the Y-maze. Other treatments consisted of applying 250 µl of footprint hexane extracts as a series of drops along the entirety of the treatment arm (approximately equivalent exposure to that of the footprint extract of the petri dish).

Field Aggregation Bioassay

Field tests were performed within a *H. convergens* overwintering macrosite at Mount San Antonio, Los Angeles County, CA, USA (elev. 1,800 m) adjacent to a level clearing where no beetles had been observed aggregating during the past three overwintering seasons (Wheeler, personal observation). In September 2012, just before the mass arrival of migrants, a grid of 16 grey cement titles (30.5 x 30.5 x 1.5 cm), spaced 4 m apart, was installed in this clearing. The bottom tile was buried into the ground so that its top face was flush with the soil surface. Two wooden stakes (40.5 x 5.0 x 0.7 cm) were laid across each tile in a '+' formation, with a grey

rubber septum placed on top of the junction. Steel legs, 1.5 cm in length, held another cement tile directly over the partially buried tile, as a roof (Fig. 6).

A Latin square design was used to distribute 4 treatment combinations across all 16 artificial hibernacula. Treatments consisted of: a septum impregnated with IBMP, a line of footprint extract painted on two stakes, treating both the septum and the stakes, and a solvent control. Septa were treated by applying 50 µl of a solution of IBMP (0.4 ng/µl, 20 ng total) to the septum well. This methoxypyrazine dose elicited aggregations in a previous field bioassay (Wheeler and Cardé, 2013). The stakes were treated by applying 2.5 ml of the footprint extract in a line down the top side of each stake. In all occurrences where either the stakes or septa were untreated, an equivalent type and quantity of clean solvent was applied. Every 2 days, the numbers of beetles at each hibernaculum were counted from photographs and treatments were refreshed until a total of 11 recordings were made over 20 days.

In order to confirm the behavioral relevance of individual hydrocarbons, the field experiment was repeated the following overwintering season (September-October 2013) on an additional set of four artificial hibernacula. During the 8-day sampling period of this trial, elevated day-time temperatures and relative humidity (average 7° C and 25 RH% increase in previous 15-day running average) likely accounted, in part, for the daily dispersal and reformation of aggregations throughout the local area. One of each of the following treatments was applied to the four artificial hibernacula: footprint extract; synthetic C_{23}; IBMP in conjunction with C_{23}, and a solvent control. Every 2 days, the numbers of beetles at each hibernaculum were counted and treatments were refreshed until a total of 4 sets of recordings were made over the course of 8 days.

Results

Chemical nature of extracts

All three beetle extracts (whole, legs, footprint) contained a similar array of saturated and unsaturated hydrocarbons (identified by characteristic dominant ions and the molecular ion from mass spectra). These included 8 monoenes, 8 dienes, and 1 possible triene or ring structure, with 23, 25, 27, 29, 31, 33, and 35 carbon chain lengths (Fig. 7, Table 2). Fractionating the extracts on a silver nitrate-impregnated silica gel column separated the unsaturated from the saturated hydrocarbons (Fig. 8). Because the saturated hydrocarbon fraction proved to be active in the Y-maze bioassay (see following subheading), the hydrocarbons it contained were quantified (Table 3) for preparation of a synthetic blend mimicking the insect-produced compounds.

Y-maze Bioassay

More diapausing beetles oriented to the arm of the Y-maze that had been pre-exposed to conspecifics than to the control arm (Table 4). Similarly, the footprint extract also was significantly preferred over the control arm of the Y-maze. Conversely, significantly more non-diapausing females oriented to the control arm, when it was presented in conjunction with either the pre-exposed or footprint-extract treatments. In contrast, non-diapausing male beetles still showed a preference for the treated arm.

These initial Y-maze bioassays indicated an orientation preference for a surface that had previously been exposed to the general walking/aggregative activity of conspecifics. Subsequent Y-maze bioassays narrowed down the list of bioactive compound(s) by testing fractions of the crude footprint extract and synthetic blends of the compounds it contains. When given the choice between an arm treated with the saturated hydrocarbon fraction or the crude footprint

extract and either a blank arm or an arm treated with the unsaturated hydrocarbon fraction, diapausing beetles preferred the saturated alkanes treatment arm (Table 5). Beetles showed no preferences between the unsaturated hydrocarbon fraction and the control. These results indicate that one or multiple components of the 5 hydrocarbons found in the saturated fraction are responsible for the orientation preference.

We tested all treatment combinations for significant differences between the amounts of time it took beetles to 'choose' (or orient up) one treatment arm compared to another (Fig. 9). Shapiro-Wilk tests indicated that the latencies for all treatments were normally distributed. The average latency to choosing the saturated hydrocarbon treatment was significantly shorter; paired with either the control arm (GLM: F_{40}=29.84, P<0.001), or the unsaturated hydrocarbon treatment (GLM: F_{40}=23.27, P<0.001). There was no significant difference in latency between the blank and unsaturated treatment arms (GLM: F_{40}=0.76, P=0.39), nor the crude footprint extract and saturated treatment arm (GLM: F_{40}=1.44, P=0.24). To control for the 4 multiple tests, the p-value can be set at 0.05 / 4 = 0.0125. The original treatment combination of crude footprint extract and a solvent control were repeated and tested intermittently throughout the other treatment combination trials as a positive control for beetles' normal aggregative behavior.

The choice arm treated with a synthetic blend of the 5 saturated hydrocarbons (same ratio and concentration as those from the saturated fraction) was "chosen" significantly more often than the choice arm treated with clean solvent (Table 6). Removing the minor components from the synthetic blend (C_{24} and C_{26}) did not affect this preference. Omitting C_{23} from the synthetic blend, however, removed any significant "preference" for the HC-treated arm. There was no significant difference in preference between an arm treated with C_{23} alone, and an arm

treated with the footprint extract, indicating that C_{23} was the primary and possibly sole bioactive component.

Field Aggregation Bioassay

Migrating beetles entered and accumulated in the artificial hibernacula (Fig. 10). The number of beetles inhabiting each artificial hibernaculum was affected by the chemical treatment (autoregressive repeat measures GLM: $F_3 = 54.97$, $P<0.0001$), the day sampled ($F_{10} =9.24$, $P<0.0001$), and the interaction between these two variables ($F_{30} =6.28$, $P<0.0001$). Similar to results from Wheeler and Cardé (2013), the methoxypyrazine treatment (IBMP) initially contained more migrant beetles than controls (initial positive slope of $Y=11.03x–11.73$ [$R^2 =0.9981$] reaching a mean of 43.25 ± 4.85SE beetles on the 8th day). Aggregations at IBMP-treated hibernacula subsequently dwindled in size and by day 12 there were no significant differences between these and the controls. The hibernacula baited solely with footprint extracts showed a longer delay before eventually amassing an average of 40.0 ± 17.2 SE beetles at 14 days, but these too disbanded over time and were the same size as the controls by day 18. In contrast, hibernacula baited with a combination of IBMP and the footprint treatments experienced an initial amassing of beetles similar to the IBMP only treatment (no significant difference between IBMP and combination treatments during the first 10 days), with continued growth in number over the course of the experiment ($Y=8.96x + 2.6$, $R^2 = 0.9274$; autoregressive repeat measures GLM: $F_{10} =24.77$, $P<0.0001$). Statically similar numbers of beetles accumulated in the footprint and combination treatments during a peak in activity at the hibernacula around day 14 (40±8.6 and 68±7.3 SE respectively). Square-root transformation of the count data was conducted due to unequal variances (larger variance between replicates with higher beetle counts).

Field confirmation of C_{23} functions

The number of beetles recorded at each artificial hibernaculum was not auto-correlated across the 4 consecutive sampling periods (as in the previous longer-term field bioassay that was conducted earlier in the migration season). Totals for each treatment were thus averaged over all sampling periods (Fig. 11). Significantly more beetle aggregated at all odor-treated hibernacula, compared to those at the blank controls (GLM: F_{16}=15.59, P<0.01). There were no differences between the numbers of beetles that aggregated at the hibernacula treated with the footprint extract versus the ones treated with synthetic C_{23}. Artificial hibernacula treated with a combination of C_{23} and IBMP had larger aggregations than those treated with the hydrocarbon alone, but the difference was non-significant (GLM LS means Tukey test: P=0.74, N=16).

Discussion

We found that tricosane (C_{23}), a *H. convergens* cuticular hydrocarbon, contributes to the formation and longevity of overwintering aggregations. A methoxypyrazine also affects aggregation behavior, but may be most effective at maintaining aggregations at novel microsites when presented in conjunction with C_{23}. The C_{23} hydrocarbon is deposited on surfaces traversed by *H. convergens.* As migrants arrive at the overwintering site, the active deposition of C_{23} at potential hibernacula may be considered a pheromone, because the signaler likely benefits from aggregative responses of conspecifics. However, it is difficult to imagine how an individual signaler could benefit from leaving this persistent C_{23} as a site-marker for future generations of migrants. We propose that C_{23} functions as a pheromone during the same overwintering season in which conspecifics are depositing it on hibernacula surfaces, but functions as a cue when it affects the orientation of future generations of migrants to the same hibernaculum.

The epicuticular hydrocarbons of some coccinellids are known to match those deposited by the tarsi (Kosaki and Yamaoka, 1996). Similarly, extracts of the *H. convergens* cuticle as well as the residues left as they walk on substrates consist largely of the same hydrocarbons at similar ratios ($C_{27:1}$, $C_{30:1}$, and $C_{32:2}$ were at very low concentrations and were not detected in all extracts [Table 2]). Analyses of the extracts showed a series of n-alkanes and saturated hydrocarbons ranging from C_{23} to C_{35}. As expected from a typical insect hydrocarbon profile, the even numbered hydrocarbon chains make up a very small percentage of the blend (Blomquist, 2010). Both extracts contained the biologically active C_{23} as a major component. We suggest that this hydrocarbon is transferred passively from the cuticle that makes contact with the surface, and functions as an unspecific "footprint cue." Similarly, foraging bumblebees (*Bombus terrestris*) avoidance of recently visited (and nectar-depleted) flowers was found to be due to the passive contamination of flowers with insect residues rather than active labeling of the flower corolla with repellent pheromones. Conspecifics were instead responding to the incidental deposition of blends of hydrocarbons (alkanes and alkenes, C_{23} to C_{31}) by the tarsi of previous foragers of the same or different species (Wilms and Eltz, 2008). This hydrocarbon blend was the same blend that accumulates at nest entrances, where it serves in kin recognition, and thus it was suggested that at the flower the compounds were not specifically released pheromones, but instead were deposited unspecifically wherever bees walk.

From a receiver's perspective, the evolution of communication may begin when receivers develop a behavioral response to conspecifics' unintentional release of metabolic, reproductive, and host byproducts (Wertheim et al., 2005). A sender could not evolve a novel aggregation pheromone without some confidence that it will serve to form an aggregation; just

as the receiver likely has no *a priori* way of associating a novel compound with any benefit. In the case of an aggregation pheromone like C_{23} produced by *H. convergens*, it may have evolved as a cue for aggregations because it was a reliable indication of conspecific presence, or it might function as an indirect measure of habitat quality (Greene and Stamps, 2001), or simply as an alternate locational cue used to increase the efficiency at which otherwise inconspicuous, or highly dispersed habitat patches are located (Childress and Hernkind, 2001). The latter seems likely because these hidden overwintering microsites are devoid of food resources and isolated within an apparently unsuitable habitat matrix far from summer foraging grounds.

If persistent aggregation cues are left behind at hibernacula by previous generations of conspecifics, then conceivably the site would attract others regardless of changes in the habitat or prevailing resident densities. This would explain why the treated artificial shelters installed in the field were able to attract sizable aggregations despite lacking the microclimatic conditions and pre-existing group membership of the adjacent traditionally-used natural hibernacula. Persistent aggregation cues would be an external form of social memory, that facilitate the long-term maintenance of aggregation sites without requiring individuals to express site fidelity (Donaldson and Grether, 2007). As subsequent visitors also label the site, a strong form of positive feedback likely persists, further establishing the microsite as a hibernaculum, leading to some degree of 'cultural inertia' (Donaldson and Grether, 2007; Boyd and Richerson, 1985). The addition of persistent aggregation pheromones from these conspecifics increases the influential pattern of conspecific dispersal and adds another layer of organization overlaying the template formed by resource heterogeneity (Dussutour et al., 2005), often leading to the use of traditional sites (Donaldson and Grether, 2007). In this way, a species preference for a particular

habitat type is amplified by the simultaneous attraction to other conspecifics also at that site (Deneubourg et al., 2002). Without persistent site-labeling, no pattern of consistent reuse of the same hibernacula would be expected (Donaldson and Grether, 2007).

Given the need for a persistent cue, it is not surprising that the C_{23} hydrocarbon turned out to be the most abundant of the saturated hydrocarbons among *H. convergens* cuticular compounds. Saturated hydrocarbons are not readily degraded and are thus much more stable than their unsaturated counterparts (Hemptinne et al., 2001). A recent study of overwintering *H. axyridis*, however, suggests that based on their relative abundance, it might be the unsaturated and/or methyl-branched hydrocarbons that are primarily responsible for the formation of conspecific aggregations (Durieux et al., 2013). Whereas unsaturated hydrocarbons may not be able to persist on aggregation surfaces as efficiently as their saturated counterparts, their variation in the placement of double bonds facilitates the potential to communicate a lot more information, such as species-specificity (Blomquist, 2010).

C_{23} is one of the shorter chain hydrocarbons found in *H. convergens* cuticular extracts, and can be considered to be semivolatile (Bartelt, 2010). This "semi-volatility" suggest that it could also be detected through olfaction, and thus mediate medium-range behavioral responses, or at least responses that do not necessarily require physical contact with the marked surface. The repellent hydrocarbon cues left by *B. terrestris* consist of alkanes and alkenes of 23 to 31 carbons in length and are perceived without the bee alighting on the surface of the flower (Wilms and Eltz, 2008). Rove beetles, *Aleochara curtula*, release a series of alkenes as olfactory pheromones involved in mating (Z7 and Z9 heneicosene, and Z7 and Z9 tricosene), as do the tenebrionid beetles, *Parastizopus transgariepinus* (1-tridecene) (Bartelt, 2010). The

greater volatility of the behaviorally active C_{23}, as compared to longer chains, may improve the chances that migrant *H. convergens* would perceive these aggregation cues and locate widely dispersed aggregation sites. This would be particularly important for beetles orienting to hibernacula that had not been used since the previous year, by which time the much more volatile methoxypyrazine markers would have dissipated.

The probability of locating an inconspicuous hibernaculum is improved by possible interactions between the responses to C_{23} and those to the more volatile, yet persistent (Woolfson and Rothschild, 1990), methoxypyrazine pheromones. Orientation to hibernacula likely takes place over many spatial scales and sensory modes. While visual orientation to vertically prominent objects likely operates initially among flying migrants at the largest spatial scales (Nalepa et al., 2005), orientation to methoxypyrazines likely operates at intermediate distances, directing migrants to the general overwintering macrosite. From there, tactile responses to environmental conditions of microhabitats (Hagen, 1962; Copp, 1983; Hodek and Honěk, 1996; Nalepa et al., 2005) and behavioral responses to conspecific hydrocarbon cues may direct migrants to the precise communal hibernaculum.

The primary role of the lipid layer covering the cuticle is to protect terrestrial insects from desiccation by minimizing transpiration (Lockey, 1988). In many species, however, one class of these waxy epicuticular lipids, the hydrocarbons, have supplementary behavior-modifying properties. Cuticular hydrocarbons have a wide variety of functions, especially among social insects, including serving as signals for nestmate/kin recognition, species/gender/dominance/fertility, chemical mimicry, primer pheromones, and task-specific signals (Blomquist and Bagnéres, 2010). Depending on the context, responses to the coccinellid

hydrocarbons can vary. Previous research has highlighted how foraging Coccinellidae leave a blend of hydrocarbons on the plants on which they forage, and that this blend deters female conspecifics from ovipositing on those particular plants (Hemptinne and Dixon, 2000; Michaud and Jyoti, 2007). A similar orientation response was seen in this study among non-diapausing (sexually mature, see appendix 3.1) females, which avoided ascending the arm of a Y-maze treated with these hydrocarbons (Table 3). When these females are in diapause, however, their orientation response switches. Ovipositing females avoid conspecific semiochemicals because of the potential costs of intraguild predation and resource competition between eggs/larvae. Under the different physiological context of diapause, it is an advantage for coccinellids (especially *H. convergens*) to form expansive aggregations and thus diapausing females preferentially orient towards these semiochemicals. This behavioral shift is not in response to chemically different "attractant" or "repellent" pheromones, but more likely an adoption of opposite responses to the same chemical cue due to context-specific selective pressures (Wilms and Eltz, 2008). This alternate function of the hydrocarbon aggregation cues highlights their evolutionary origins. Chemical signals can be costly to produce and respond to, and it can be difficult to explain how the production of such costly signals could have originated and expanded before a beneficial response evolved. Some of these costs can be defrayed if those signals have other supplementary benefits (Blum, 1996), as is the case here, where *H. convergens* are responding to preexisting cuticular chemistry. These evolutionary origins, as well as any physiological constraints on biosynthetic pathways, are factors thought to be responsible for the multiple functions characterizing many semiochemicals (Blum, 1996; Wertheim et al., 2005).

Literature Cited

Arnaud, L., Spinneux, Y., and Haubruge, E. 2003. Preliminary observations of sperm storage in *Adalia bipunctata*: sperm size and number. *Applied Entomology and Zoology*. 38: 301-304.

Bartelt, R. J. 2010. Volatile hydrocarbon pheromones from beetles. In G. J. Blomquist, and A.-G. Bagnéres (Eds.), *Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology* (pp. 448-476). Cambridge, UK: Cambridge University Press.

Baust, J. G., and Miller, L. K. 1975. Supercooling phenomenon and water content independence in the overwinting beetle, Coleomegilla maculata (Col., Coccinellidae). *Journal of Insect Physiology.* 21:1751-1754.

Bennett, L. E., and Lee, R. E. 1989. Simulated winter to summer transition in diapausing adults of the lady beetle (Hippodamia convergens). *Physiological Entomology*. 14:361-367.

Benton, A. H., and Crump, A. J. 1979. Observations on aggregation and overwintering in the coccinellid beetle Coleomegilla maculata (DeGeer). *Journal of NY Entomological Society.* 87:154-159.

Blomquist, G. J. 2010. Biosynthesis of cuticular hydrocarbons. In G. J. Blomquiste, and A.-G. Bagnéres (Eds.), *Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology* (pp. 35-52). Cambridge, UK: Cambridge University Press.

Blomquist, G. J., and Bagnéres, A.-G. (Eds.). 2010. *Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology.* Cambridge, UK: Cambridge University Press.

Blum, M. S. 1996. Semiochemical parsimony in the Arthropoda. *Annual Review of Entomology.* 41:353-374.

Boyd, R., and Richerson, P. J. 1985. *Culture and the Evolutionary Process.* Chicago, IL: University of Chicago Press.

Brown, A. E., Riddick, E. W., Aldrich, J. R., and Holmes, W. E. 2006. Identification of (-)-β-caryophyllene as a gender-specific terpene produced by the multicolored Asian lady beetle. *Journal of Chemical Ecology*. 32: 2489-2499.

Childress, M. J., and Hernkind, W. F. 2001. The guide effect influence on the gregariousness of juvinile Caribbean spiny lobsters. *Animal Behaviour.* 62:465-472.

Copp, N. H. 1983. Temperature-dependent behaviours and cluster formation by aggregating ladybird beetles. *Animal Behaviour.* 31:424-430.

Cudjoe, E., Wiederkehr, T. B., and Brindle, I. D. 2005. Headspace gas chromatography-mass spectrometry: a fast approach to the identification and determination of 2-alkyl-3-methoxypyrazine pheromones in ladybugs. *Analyst.* 130:152-155.

Deneubourg, J.-L., Lioni, A., and Detrain, C. 2002. Dynamics of aggregation and emergence of cooperation. *Biological Bulletin.* 202:262-267.

Donaldson, Z. R., and Grether, G. F. 2007. Tradition without social learning: scent-mark-based communal roost formation in a Neotropical harvestman (Prinostemma sp.). *Behavioral Ecology and Sociobiology.* 61:801-809.

Durieux, D., Fischer, C., Brostaux, Y., Sloggett, J. J., Deneubourg, J.-L., Vandereycken, A., et al. 2012. Role of long-chain hydrocarbons in the aggregation behaviour of Harmonia axyridis (Pallas) (Coleoptera: Coccinellida). *Journal of Insect Physiology.* 58:801-807.

Durieux, D., Fassotte, B., Vanderplank, M., Deneubourg, J-L., Fischer, C., Lognay, G., Haubruge, E., and Verheggen, F. J. 2013. Substrate marking by an invasive ladybeetle: Seasonal changes in hydrocarbon composition and behavioural responses. *PLoS One.* 8:e61124.

Dussutour, A., Deneuboug, J., and Fourcassie, V. 2005. Amplification of individual preferences in a social context: The case of wall-following in ants. *Proceedings of the Royal Society of Biological Sciences.* 272:705-714.

Evans, E. W. 2004. Habitat displacement of North American ladybirds by an introduced species. *Ecology.* 85:637-647.

Greene, M. C., and Stamps, J. A. 2001. Habitat selection at low population densities. *Ecology.* 82:2091-2100.

Hagen, K. S. 1962. Biology and ecology of predaceous Coccinellidae. *Annual Review of Entomology.* 7:289-326.

Hemptinne, J. L., and Dixon, A. F. 2000. Defence, oviposition and sex: semiochemical parsimony in two species of ladybird beetles (Coleoptera, Coccinellidae)? A short review. *European Journal of Entomology.* 97:443-447.

Hemptinne, J. L., Lognay, G., Doumbia, M., and Dixon, A. F. 2001. Chemical nature and persistence of the oviposition deterring pheromone in the tracks of the larvae of the two spot ladybird, *Adalia bipunctata* (Coleoptera: Coccinellidae). *Chemoecology.* 11:43-47.

Hemptinne, J. L., Lognay, G., Gauthier, C., and Dixon, A. F. 2000. Role of surface chemical signals in egg cannibalism and intraguild predation in ladybirds (Coleoptera: Coccinellidae). *Chemoecology* 10:123-128.

Hemptinne, J.-L., and Dixon, A. F. 2000. Defence, oviposition and sex: semiochemical parsimony in two species of ladybird beetles (Coleoptera, Coccinellida)? A short review. *European Journal of Entomology.* 97:443-447.

Hodek, I., and Honêk, A. 1996. *Ecology of Coccinellidae.* Boston: Kluwer Academic.

Honěk, A., Martinková, Z., and Pekár, S. 2007. Aggregation characteristics of three species of Coccinellida (Coleoptera) at hibernation sites. *European Journal of Entomology.* 104:51-56.

Kosaki, A., and Yamaoka, R. 1996. Chemical composition of footprints and cuticular lipids of three species of lady beetles. *Jpn J Appl Entomol Zool*. 40:47-53.

Lockey, K. H. 1988. Lipids of the insect cuticle: origin, composition and function. *Comp. Biochem. Physiol.* 89B:595-645.

Majerus, M. E. N. 1997. How is *Adalia bipunctata* (Linn.) (Coleoptera: Coccinellidae) attracted to overwintering sites? *The Entomologist.* 116:602-615.

Michaud, J. P., and Jyoti, J. L. 2007. Repellency of conspecific and heterospecific larval residues to *Hippodamia convergens* (Coleoptera: Coccinellidae) ovipositing on sorghum plants. *European Journal of Entomlogy.* 104:399-405.

Mishra, G., and Omkar. 2006. Conspecific interference by adults in an aphidophagous ladybird *Propylea dissecta* (Coleoptera: Coccinellidae): effect on reproduction. *Bulletin of Entomological Research.* 96: 407-412.

Nalepa, C. A., Kennedy, G. G., and Brownie, C. 2005. Role of visual contrast in the alighting behavior of Harmonia axyridis (Coleoptera: Coccinellidae) at overwintering sites. *Environmental Entomology.* 34:425-431.

Omkar, Pervez, A., and Gupta, A. K. 2004. Role of surface chemicals in egg cannibalism and intraguild predation by neonates of two aphidophagous ladybirds, *Propylea dissecta* and *Coccinella transversalis*. *Journal of Applied Entomology.* 128: 691-695.

Pettersson, J., Ninkovic, V., Glinwood, R., Birkett, M. A., and Pickett, J. A. 2005. Foraging in a complex environment - semiochemicals support searching behaviour of the seven spot ladybird. *European Journal of Entomology.* 102:365-370.

Rankin, M. A., and Rankin, S. 1980a. Some factors affecting presumed migratory flight activity of the convergent ladybeetle, *Hippodamia convergens* (Coccinellida, Coleoptera). *Biological Bulletin.* 158:356-369.

Rankin, S. M., and Rankin, M. A. 1980b. The hormonal-control of migratory fight behaviour in the convergent ladybird beetle, *Hippodamia convergens*. *Physiological Entomology*. 5:175-182.

Ruzicka, Z. 2001. Oviposition responses of aphidophagous coccinellids to tracks of ladybirds (Coleoptera: Coccinellidae) and lacewing (Neuroptera: Chrysopidae) larvae. *European Journal of Entomology.* 98:183-188.

Seagraves, M. P. 2009. Lady beetle oviposition behavior in response to the trophic environment. *Biological Control.* 51:313-322.

Steinmetz, I., Schmolz, E., and Ruther, J. 2003. Cuticular lipids as trail pheromone in a social wasp. *Proceedings of the Royal Society B Biological Sciences.* 270:385-391.

Susset, E. C., Ramon-Portugal, F., Hemptinne, J.-L., Dewhirst, S. Y., Birkett, M. A., and Magro, A. 2013. The role of semiochemicas in short-range location of aggregation sites in *Adalia bipunctata* (Coleoptera, Coccinellidae). *Journal of Chemical Ecology.* 39:592-601.

Symonds, M. R., and Elgar, M. 2008. The evolution of pheromone diversity. *Trends in Ecology and Evolution.* 23:220-228.

Wertheim, B., van Baalen, E.-J. A., Dicke, M., and Vet, L. E. 2005. Pheromone-Mediated Aggregation in Nonsocial Arthropods: An Evolutionary Ecological Perspective. *Annual Review of Entomology.* 50:321-346.

Wheeler, C. A., and Cardé, R. T. 2013. Defensive allomones funciton as aggregation pheromones in diapausing ladybird beetles, *Hippodamia convergens*. *Journal of Chemical Ecology.* 39:723-732.

Wilms, J., and Eltz, T. 2008. Foraging scent marks of bumblebees: footprint cues rather than pheromone signals. *Naturwissenschaften.* 95:149-153.

Woolfson, A., and Rothschild, M. 1990. Speculating about pyrazines. *Proceedings of the Royal Society of London: B Biology.* 242:113-119.

Tables and Figures

Table 2.

Identification and relative amounts of chemicals present in three different sources of *Hippodamia convergens* extracts. Ion fragmentation patterns in mass spectra were characteristic of non-branched hydrocarbons, and thus molecular ions were used to make tentative identifications. The values after the colon in the 2nd column indicate the number of double bonds present. The compounds that were not detected in extracts are indicated with a dash. Amounts are given as percentages relative to the most abundant compound, C_{29}:1.

Peak number	Hydrocarbon chain length	Retention time	Molecular Ion	Footprint extracts	Whole Body extracts	Leg extracts
1	**C_{23}**	15.16	324	32.9	36.98	22.4
2	C_{24}	16.97	338	0.84	1.5	0.49
3	**C_{25}**	18.88	352	16.18	21	11.8
4	C_{25}:1	19.18	350	13.84	8.52	12.54
5	C_{26}	20.71	366	1.37	2.47	0.73
6	C_{27}:1	22.48	378	1.64	--	2.37
7	**C_{27}**	22.61	380	21.39	25.75	22.14
8	C_{27}:1	22.77	378	54.77	40.61	52.03
9	C_{28}:1	24.41	392	3.32	0.65	3.3
10	C_{29}:1	26.05	406	28.44	23.14	25.27
11	C_{29}:1	26.25	406	100	100	100
12	C_{29}:2	26.54	404	8.98	6.67	7.65
13	C_{30}:1	27.75	--	--	--	2.03
14	C_{30}:2	27.85	418	2.09	1.68	3.08
15	C_{31}:1	29.55	434	55.54	33.77	85.05
16	C_{31}:2	29.57	432	72.13	92.9	69.61
17	C_{32}:2	31.03	446	2.07	--	3.71
18	C_{33}:2	32.53	460	36.43	21.69	55.23
19	C_{33}:2	32.64	460	69.32	65.6	70.48
20	C_{35}:2	35.82	488	5.54	2.79	9.56
21	C_{35}:2	35.91	488	3.69	1.3	5.8

Table 3.

Identification and quantification of saturated hydrocarbons isolated from the crude footprint extract of *Hippodamia convergens*. An internal standard of a similar hydrocarbon, 5-methyl-heptacosane, was used for quantification. Quantification of the concentration at which these footprint compounds are naturally applied was also calculated by factoring in the area of the petri dish from which the extract was collected.

Peak number	Hydrocarbon chain length	Concentration (ng/µl)	Concentration (ng/mm^2)
1	C_{23}	40.9	24.6
2	C_{24}	2.0	1.2
3	C_{25}	22.3	13.4
4	C_{26}	2.9	1.7
5	C_{27}	24.0	14.5
6	$5MeC_{27}$ ISTD	95.0	n/a

Table 4.

Chi-squared test of the proportion of total responding *Hippodamia convergens* that choose the treated arm over the control arm in Y-maze bioassays. Treatments included 'pre-exposing' the surface to aggregating beetles *in vivo*, or 250 µl of footprint extract. *N*=30.

Treatment arm	Male		Female	
	%	P	%	P
Diapause / Unmated				
Pre-exposed	70	< 0.05	67	0.068
Footprint extract	83	< 0.05	77	< 0.05
Summer foraging / Mated				
Pre-exposed	77	< 0.05	20	0.001
Footprint extract	87	< 0.001	13	< 0.001

Table 5.

Chi-squared test of the proportion of total responding *Hippodamia convergens* that choose a particular arm of the Y-maze bioassay. Four different treatment combinations were conducted consisting of solvent controls, the crude footprint extract, the saturated alkanes fraction of the extract, and the unsaturated alkanes fraction of the extract. Treatments in bold indicate the preferred treatment arm. *N*=40.

Treatment Choices	%	p	X^2
Saturated vs.	82.5	**<0.0001**	16.9
Blank	17.5		
Unsaturated vs.	45		
Blank	55	0.53	0.4
Saturated vs.	75	**0.0016**	10.0
Unsaturated	25		
Saturated vs.	40		
Crude	60	0.21	1.6

Table 6.

Chi-squared test of the proportion of total responding *Hippodamia convergens* that choose different synthetic blends of hydrocarbons (HC) identified from the saturated alkanes fraction of footprint extracts. The order in which HCs were subtracted from the blend is shown in the order of the treatment rows listed below. Treatments in bold indicate significant preference. *N* = 30.

Treatment Choices	%	*P*	X^2
C_{23}, C_{24}, C_{25}, C_{26}, C_{27}	80	**0.0010**	10.8
blank	20		
C_{23}, C_{25}, C_{27}	76.7	**0.0035**	8.5
blank	23.3		
C_{25}, C_{27}	60	0.27	1.2
blank	40		
C_{23}, C_{27}	73.3	**0.011**	6.5
blank	26.7		
C_{23}	70	*0.029*	4.8
blank	30		
C_{23}	36.7		
crude	63.3	0.14	2.1

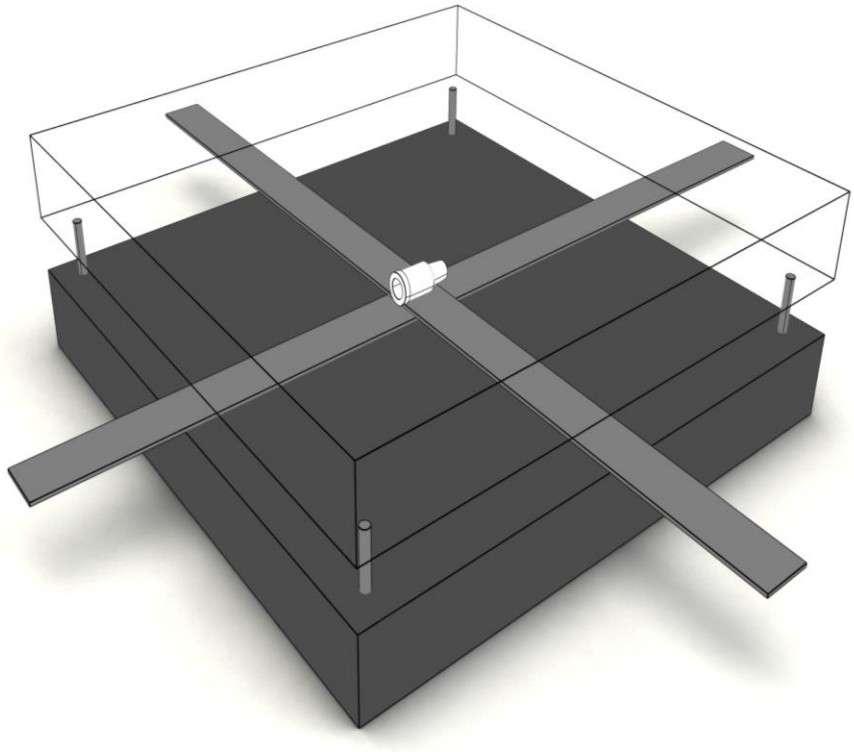

Figure 6. Diagram of artificial hibernaculum used in field bioassays. The wooden stakes (light grey; 40.5 x 5.0 x 0.7 cm) were treated with hydrocarbon treatments and laid in an "+" formation on the bottom square cement tile (dark grey; 30.5 x 1.5 cm). The septum placed at the junction of the crossed stakes was baited with 2-isobutyl-3-methoxypyrazine. The top cement tile roof, rendered transparent in this diagram, is held 1.5 cm above the bottom tile.

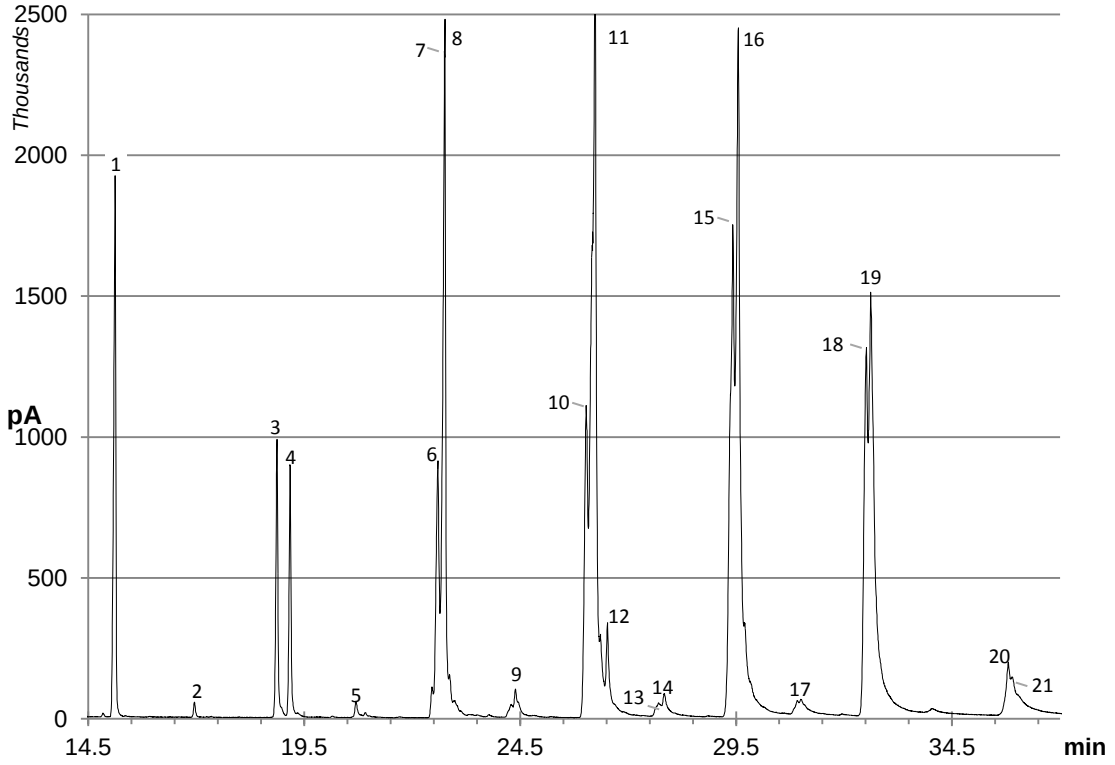

Figure 7. Gas chromatogram of the footprint extract from *Hippodamia convergens*. The chromatogram depicts straight-chain saturated and unsaturated hydrocarbons ranging from 23 to 35 carbons in length. Numbers above peaks match those in Table 2.

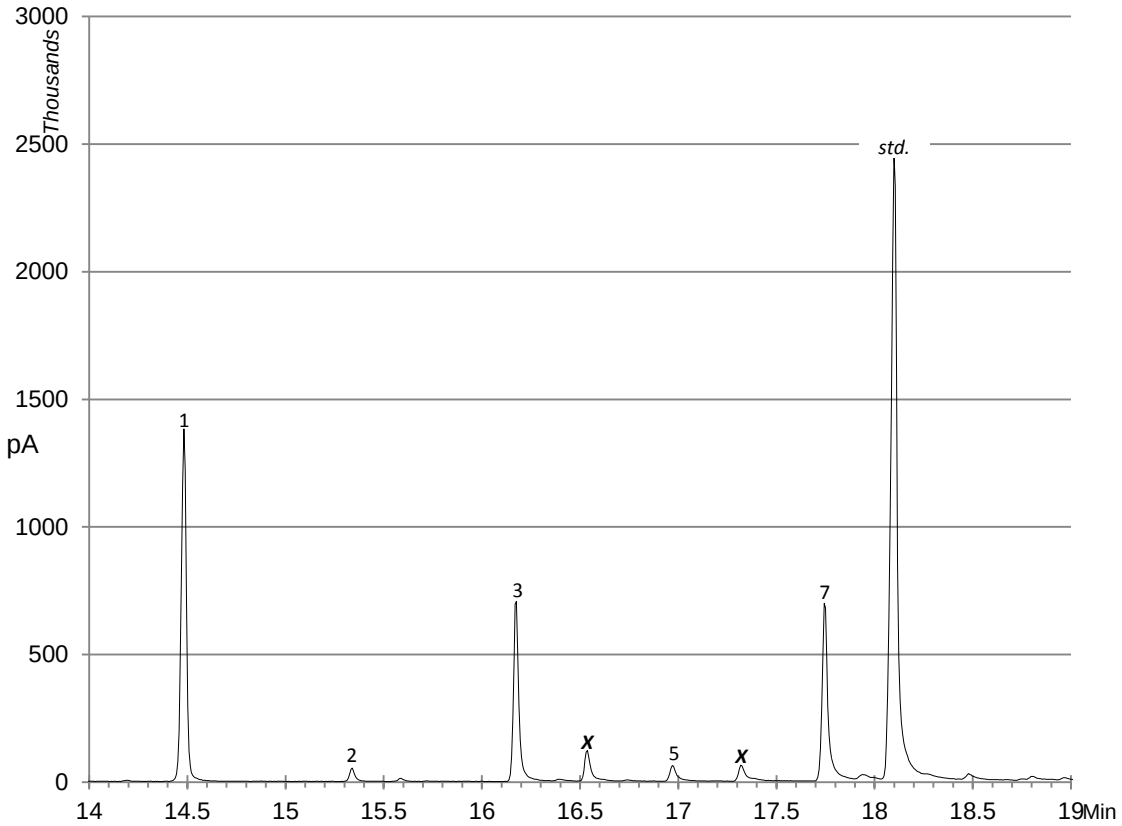

Figure 8. Gas chromatogram from the saturated hydrocarbon fraction of the *Hippodamia convergens* crude footprint extract shown in Fig. 7. Extract contains a known concentration of 5-methyl-heptacosane as an internal standard at peak marked std. The two peaks marked with an "X" were identified as contaminants ($5MeC_{25}$ and $5MeC_{26}$) from the internal standard and were omitted from calculations on quantity. Numbers above each peak match those in Table 2.

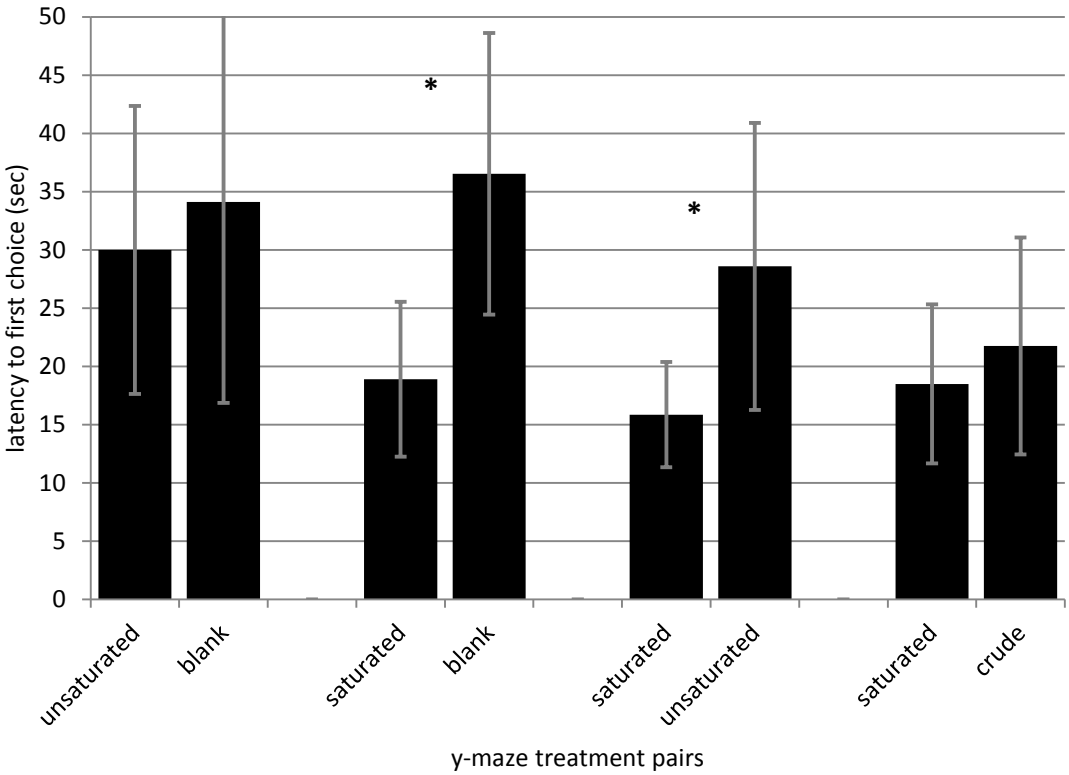

Figure 9. Histogram of *Hippodamia convergens'* mean latencies until making first "choices" of a Y-maze treatment arm among four different pair-wise treatment combinations. Treatment combinations included arms treated with crude footprint extract, the saturated hydrocarbon fraction, the unsaturated hydrocarbon fraction, or a solvent blank. Error bars show standard deviation, and the '*' symbol indicates significant differences **within** each treatment pair (GLM LS means Tukey test: $P<0.05$, $N=40$).

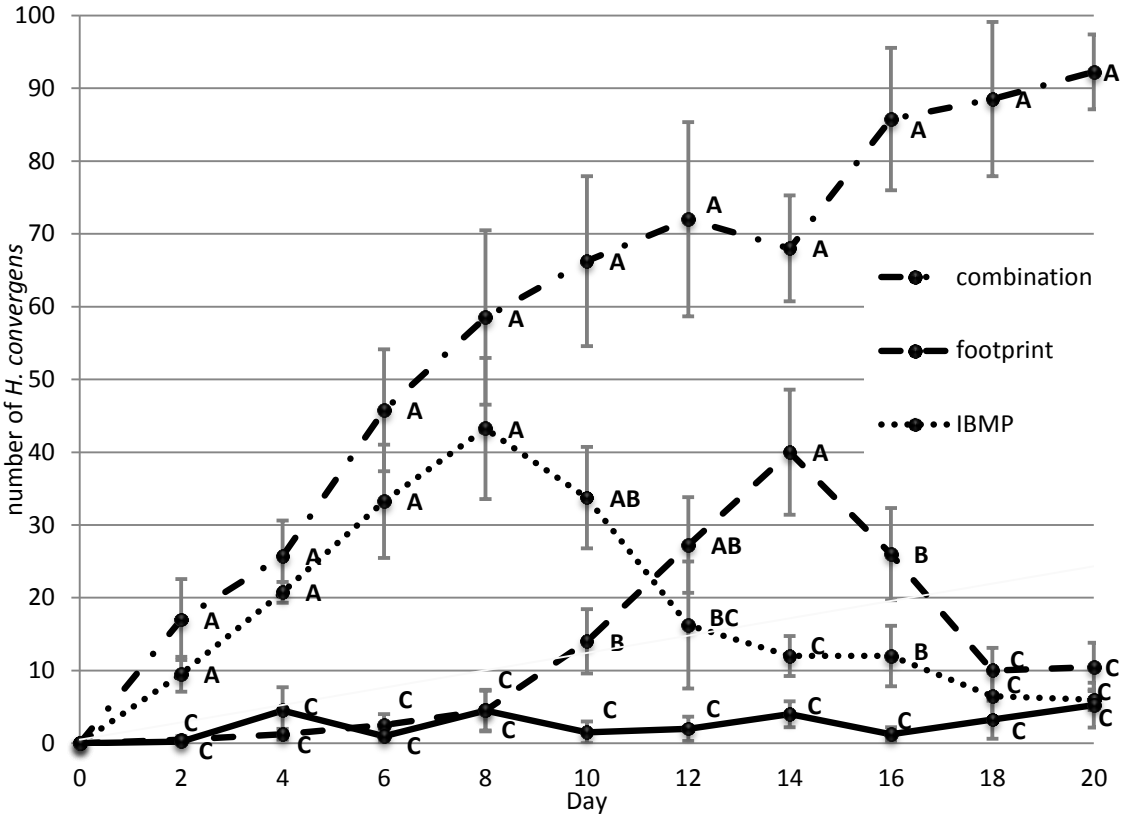

Figure 10. Mean numbers of *Hippodamia convergens* in 'baited' artificial hibernacula over the course of 20 days. Treatments included footprint extract (footprint), IBMP, and a combination of both treatments (combination). Error bars show standard errors among the 4 hibernacula replicates, and different letters indicate significant differences among treatments for each day (GLM LSmeans Tukey test: $P<0.05$, $N=4$).

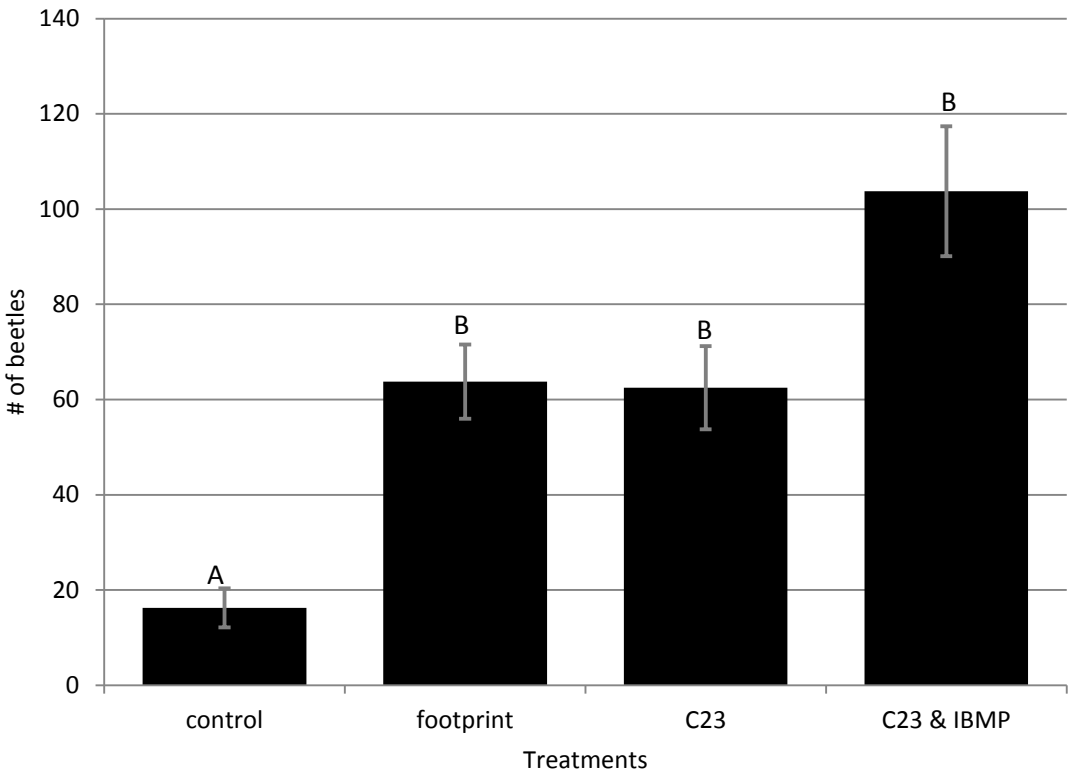

Figure 11 Histogram of the mean number of *Hippodamia convergens* found aggregating in artificial hibernacula treated with either solvent (control), footprint extract (footprint), C23 hydrocarbon, or both C23 hydrocarbon and IBMP. Hibernacula were sampled 4 times between October and November, 2012. Letters indicate significant differences ($P < 0.05$). N=4.

Appendices

Appendix 3.1 Transition of pre-reproductive diapausing females to reproductive maturity.

Introduction and Methods

Post-teneral, pre-reproductive coccinellid female migrants arrive at overwintering sites in a state of reproductive diapause (Rankin and Rankin 1980a). Lengthening photophases and warmer conditions at these overwintering sites, especially near the end of the overwintering season, stimulate mating behavior (Benton and Crump, 1979; Copp, 1983; Honêk et al. 2007). Access to live prey stimulates ovarian development and oviposition, and thus terminates diapause (Rankin and Rankin, 1980a; Hodek and Honěk, 1996; Michaud and Qureshi, 2005). The reproductive maturity of diapausing and non-diapausing *H. convergens* was compared by observing sperm storage, oviposition behavior, and ovarian development during a simulated overwintering to summer transition.

Diapausing *H. convergens* were collected from an overwintering aggregation. Half of these overwintering beetles (≈50) were held in an environmental chamber that maintained them in a state of diapause (as described in Wheeler and Cardé, 2013). The other half of the overwintering beetle were transferred to a greenhouse programmed to mimic conditions known to terminate diapause (16L:8D photoperiod, 20-27° C and 68% RH [Rankin and Rankin 1980; Bennett and Lee, 1989]. These beetles were fed an excess of pea aphids, *Acyrthosipohon pisum*, reared on fava bean plants, *Vicia faba*. After one month a sample of 20 females were collected from both environmental treatments and were dissected for analysis of sexual maturity.

The female beetles were dissected under a dissecting microscope. Each dissected beetle was contained in a Petri dish coated with Sylgard® and bathed in a saline ringer (described in

Collings, 1966). Lipid deposits, respiratory tubules, and internal organs within the abdominal cavity were removed with forceps and flushes of ringer solution, exposing the entire reproductive tract. One drop of Trypan blue was added and the general appearance of ovaries was noted before the spermatheca was cut at the base, removed from the beetle and placed on a hemocytometer with 3 µl of distilled water. A single squeeze was applied to the entire length of the submerged spermatheca with modified forceps. A cover slip was placed over the material on the hemocytometer, while the sperm count was achieved using a compound light microscope. Sperm were counted in the standard five of twenty-five boxes on the hemocytometer (i.e. upper leftmost, upper rightmost, center, lower leftmost, and lower rightmost boxes). To ensure unbiased cell counts, sperm that laid on the upper and lower boundaries were tallied, while sperm cells that laid on the right and left boundaries were not. Sperm counts were calculated by averaging the number of sperm cells tallied from each of the standard five 0.10 mm^2 squares of the hemocytometer cross section. The mean sperm cells per 0.10 mm^2 were multiplied by twenty-five, producing the average number of sperm cells per 1.00 mm^2.

Results and Discussion

Diapausing female beetles had a lower sperm composition in the spermatheca than those females transferred to the "summer" conditions of the greenhouse (Fig. 12). All but four of these diapausing females had empty spermatheca (n=20), while all greenhouse (presumably non-diapausing) females were storing sperm (*n*=17; three dissections failed due to experimenter error); results comparable to Arnaud et al. (2003). The females maintained in diapause were exposed to cold temperatures (5-13^{o} C) and were usually quiescent, thus greatly reducing the

possibility of mating. The beetles held in the greenhouse were very active by comparison. Female and male beetles had access to each other and pairs were frequently observed in *copula*. Multiple mating *ad libitum* would allow females to replenish the number of sperm stored in their spermathecae following egg fertilization (Arnaud et al., 2003). Within 20 days *H. convergens* eggs were observed on the *V. faba* plants provided. The ovaries of the greenhouse females appeared more mature than those of the diapausing females; oocytes were visible, and ovarioles were more plentiful and longer.

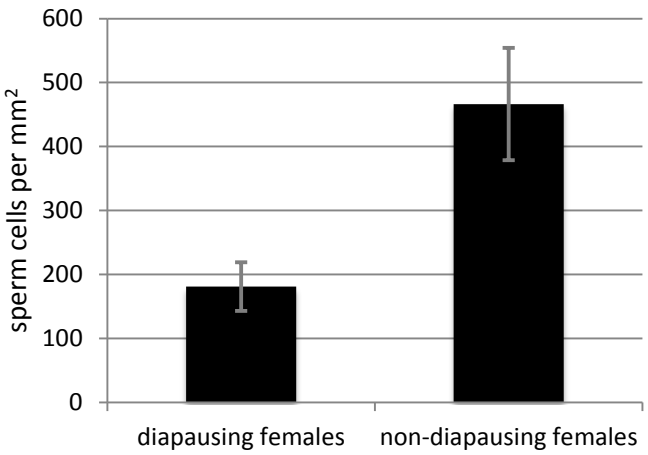

Figure 12 Comparison of the mean number of sperm cells per mm^2 in the spermatheca of presumably diapausing and non-diapausing female *Hippodamia convergens*. Error bars represent the standard error of the mean. For diapausing females, n=20 and non-diapausing females, n=17. Means were compared using a Mann-Whitney U and found to be significantly different (U=62.00, p=0.0011).

In conclusion, one-month exposure to longer photophases, higher temperatures, live aphid prey, and male conspecifics was sufficient to terminate diapause in *H. convergens* female migrants. Such results are in agreement with similar experiments conducted on *H. convergens* (Michaud and Qureshi, 2005), other species of Coccinellidae (Ranking and Rankin, 1980a; Bennett and Lee, 1989; Hodek and Ceryngier, 2000; Arnaud et al. 2003;) and Curculionidae (Doležal and Sehnal, 2007).

Literature Only Cited in Appendix 3.1

Collings, S. B. 1966. An improved ringer solution for heart of Passalus beetles, *Popilius disjunctus* (Coleoptera – Passalidae). *Annals of the Entomological Society of America*. 59:972-974.

Doležal, P. and Sehnal, F. 2007. A simple method for the detection of imaginal diapause in beetles. *Journal of Applied Entomology*. 131:221-223.

Hodek, I. and Ceryngier, P. 2000. Sexual activity in Coccinellidae (Coleoptera): a review. *European Journal of Entomology.* 97:449-456.

Michaud, J. P. and Qureschi, A. 2005. Induction of reproductive diapause in *Hippodamia convergens* (Coleoptera: Coccinellidae) hinges on prey quality and availability. *European Journal of Entomology*. 102:483-487.

Chapter 4 –

Multi-modal signal interactions in the *Hippodamia convergens* aposematic system

Abstract

In an aposematic system, the warning display (e.g., color, odor, or behavior) advertises the presence and potentially the strength of the chemical defense. A strong coupling between the indicator trait and the defensive trait is therefore integral to the evolution and maintenance of aposematism. An ostensibly conflicting theory predicts that a negative correlation between the indicator and defensive trait can exist, depending on the selective pressure on defenses and the presence of multiple aposematic signals. The aposematic ladybeetle, *Hippodamia convergens*, displays a variety of aposematic signals, the combined effects of which have been rigorously tested in predator stimulus-response experiments. Intraspecific variation of investment in these multi-modal aposematic displays, however, has not been explored thoroughly. In this study, we examine complex signal theory from the perspective of the signaler, and identify significant correlations between warning coloration, warning odor, and toxicity. We show that the red coloration of *H. convergens* functions as an honest signal of alkaloid toxicity. The correlation between methoxypyrazine concentration and toxicity, however, is more nuanced. Overall, methoxypyrazine production is negatively correlated with the degree of toxicity. Thus, the reddest females contain the highest concentrations of alkaloids and some of the lowest concentrations of methoxypyrazines. The reddest males, although less toxic than their red female counterparts, on average produce significantly more alkaloids and methoxypyrazines than orange females and orange males. Within each of these groups, the negative correlation

between methoxypyrazines and alkaloid toxicity remains. The relationship between variations in these signals may be affected by the interactions of color and odor on avoidance learning in predators, as well as the supplemental aggregative function of the aposematic methoxypyrazine signal.

Introduction

Aposematism and complex signal theory

Aposematic displays often consist of several signals of different modality (chemical: taste, odor; auditory; visual: specific coloration, behavioral displays) that all serve to warn off potential predators. It is not well understood why animals invest more in producing redundant signals to communicate the same general message (Vogler and Kelley, 1998). It is expected, however, that the benefits of a second signal must outweigh its costs, and under this principle several mechanisms of complex signal evolution have been proposed (Hebets and Papaj, 2005; Skelhorn and Ruxton, 2008; Gohli and Hogstedt, 2010). Complex signaling theory dictates that there will be correlated variation between the investments in different modes of display. This can be coupled with traditional theory on aposematism that states that the evolution of the defensive suite is constrained by selective predation on less defended prey (both through display efficacy and degree of unpalatability) and production costs. This particular selective environment results in a tight coupling between the indicator trait (display) and the defensive trait (unpalatable chemical defense). Empirical testing of these theories has focused primarily on the receiver end of signaling, with most experiments studying predator responses to different combinations of aposematic displays and defenses (e.g., Marples et al., 1994). For a better understanding of the evolution of aposematism, experiments must also explore the signaler's perspective. Specifically, identifying correlated variation between aposematic signals and the defensive chemistry that they advertise may highlight how the costs of defenses, the environment, and the signaler's physiology influence the evolution of complex signals, and aposematism.

For example, a positive correlation between the investments in different modes of aposematic display would provide support for the "redundant signal" content-based hypothesis (Hebets and Papaj, 2005). This hypothesis predicts positive correlations between different signals if those signals all provide the same information and function best to reduce predation when presented in combination. If instead one signal remained relatively constant, while another varied with toxic chemical concentration, then they may serve as "multiple messages" providing different information about some aspect of signaler quality (Hebets and Papaj, 2005).

Other theories on complex displays state that if an aposematic insect increases investment in one form of anti-predator defense, it will be expected to offset those costs by a decreased investment in another form of defense (Holloway et al., 1991; Hebets and Papaj, 2008). Additional defense displays interact to enhance predators' avoidance learning and according to a model developed by Skelhorn and Ruxton (2008), as the experience needed for avoidance learning decreases there is a reduced per capita risk of predation, and thus a decreased investment in unused or inefficient defenses. Hebets and Papaj (2005) incorporated this negative correlation into a "multitasking" inter-signal interaction hypothesis.

Aposematic coccinellids

Like several other coccinellid beetles, *H. convergens* is a classic aposematic insect engaging in multimodal anti-predator displays. Many coccinellids possess toxic alkaloids, and advertise their distastefulness with warning coloration (contrasting patterns of red and black), methoxypyrazine odors, behavioral conspicuousness, and reflex bleeding (reflexive fluid containing concentrated alkaloids and methoxypyrazines (Marples et al., 1994; Hodek and Honěk, 1996; King and Meinwald, 1996). Although the effect that the interaction between these defense displays has on predator response has been described (e.g. Dolenská et al., 2009), little

is known about the signaler's production and variation of these displays. Without such an understanding we can gain only partial insight into the function of complex signaling.

There is substantial intraspecific variation in coloration among coccinellids, especially among *Harmonia axyridis*, a species with typica (red with black spots) and melanic (black with red spots) color morphs (de Jong et al., 1991, Holloway et al., 1993). Within the typica color morph of *H. axyridis,* Cai et al. (2007) suggest that orange individuals have higher concentrations of methoxypyrazines than the lighter, yellow individuals. Variation in the methoxypyrazine aposematic display suggests the possibility that there are selective pressures on its production. The methoxypyrazine aposematic signal of *H. convergens* also functions as an aggregation pheromone (Wheeler and Cardé, 2013). This supplementary function likely reduces the cost of producing methoxypyrazines as an aposematic signal alone, and may further affect the relationship between the production of other aposematic signals and defensive chemistry. Additionally, an aggregation itself is considered an aposematic display, and thus the production of methoxypyrazines is tightly interwoven into the whole coccinellid aposematic model, leading to several, potentially competing, selective pressures on their production.

The endogenous alkaloids of *H. convergens* were identified first by Tursch et al. (1974) and their structures were fully characterized by King and Meinwald (1996). *Hippodamia convergens* possess a free-base/*N*-oxide chiral pair of azaphenalene alkaloids, hippodamine and convergine (Fig. 12). Previous attempts to quantify these alkaloids in *H. convergens* eggs have proven difficult due to the N-oxide, convergine, being thermally degraded during gas chromatography-mass spectrometry (GC-MS) analysis (Slogget et al., 2009; Kajita et al., 2010). This degradation results in multiple smaller peaks on the GC that interfere with the

hippodamine peak, making quantification inaccurate. By reducing convergine to its free-base form, hippodamine, it should elute as one peak. Kokatla et al. (2011) have described methods to reduce N-oxides with a bis(pinacolato)diboron reagent $(pinB)_2$, and I adapted this method to obtain more accurate quantitation of the total alkaloids present in these beetles.

Methods and Materials

Experimental Animals

We collected adult, diapausing *H. convergens* adults from an overwintering aggregation at Mount San Antonio, Los Angeles County, CA, USA (lat: 34.24495, lon: -117.65875, elev. 1,650 m). Individual beetles were placed in 4 ml vials and stored at -25^{o}C for 8 hours before color analysis and chemical extraction. Beetles were weighed to ±0.01 mg, and sexed by morphological features of their terminal sternites (Majerus and Kearns, 1989).

Analysis of Color

The left elytron of each beetle was removed and digitally photographed through a dissecting microscope with tungsten bulb illumination and using a Canon Rebel Ti1 (1/4 s shutter speed, 400 ISO, faithful color setting, spot metering). Image analysis software (ImageJ^{tm}, version 1.6.0_51, National Institutes of Health) was used to collect average R'G'B' histogram values from the red portion of each photographed elytron. Analyzing *H. convergens* elytra with the additive color model, R'G'B', quantifies color spectra based on the amount of red, green, and blue they contain. Each R', G', and B' value ranges from 0' to 255', and acts as a Cartesian coordinate in a Euclidean space. The brightest, most saturated red possible is represented as an R'G'B' triplet: 255', 0', 0'. The distribution of histogram values was analyzed for normality, and beetles in the top and bottom 25% were grouped into two separate color categories.

Preparation of Extracts

A total of 32 samples of 10 beetles from each sex and coloration group were homogenized with 600 µl of methanol (Optima® LC/MS, Fisher Scientific, Fair Lawn NJ.) and 600 µL of distilled hexane (Optima®, Fisher Scientific) in a ground glass homogenizer (4 ml). The extract was mixed with a stir bar (~800 rpm) for 2 hours and then centrifuged for 5 min. The bottom methanol phase was collected and divided between two vials. One vial contained 400 µl and was concentrated under nitrogen for methoxypyrazines quantification, while the other vial contained 100 µl and was run through an *N*-oxide reduction procedure for alkaloid quantification. To confirm that *H. convergens* methoxypyrazines, hippodamine, and its *N*-oxide convergine partitioned into the MeOH phase, methanol solutions of a synthetic methoxypyrazine standard, sec-butyl-methoxypyrazine (Aldrich Chemical Co., Milwaukee WI.), and proxy alkaloid standards, 1-benzylpiperidine (Alfa Aesar, Ward Hill MA.) and its *N*-oxide (synthesized from the alkaloid as described by Kokatla et al., 2011), were extracted with hexanes. Gas chromatographic analysis of the MeOH layer showed that less than 2% of the methoxypyrazines, 8% of the alkaloid, and 0% of the *N*-oxide had partitioned into the hexane layer.

Before quantification of the alkaloids, convergine was reduced to its amine, hippodamine (see Kokatla et al., 2011). First, 40 µl of a methanol solution (1 ng μl^{-1}) of the internal standard (-)-sparteine (Annova Chem Inc., San Diego CA.) was added to the vial containing 100 µl of the MeOH extract of the beetles. The reduction reaction was initiated by adding 20 µl of a methanol solution containing bis(pinacolato)diboron at 4 µg μl^{-1} ($pinB_2$, Oakwood Products Inc., West Columbia SC.; ~2 times the molar equivalent of the hippodamine expected based on preliminary trials) and flushing the vial with nitrogen. The mixture was

stirred for 24 hours in a 50° C water bath. The reduction reaction was stopped by adding 40 µl of a methanol solution containing ethylenediamine at 9.5 µg μl^{-1} (Aldrich Chemical Co.; 20 times the molar equivalent of $pinB_2$ added). The reaction mixture was then back extracted with an equal volume of hexane (200 µl) and the methanol phase was collected and dried over sodium sulfate.

An internal standard of 2-methoxy-3-methylpyrazine (Aldrich Chemical Co.) was added to the other 400 µl portion of the original methanol homogenate at a concentration of 31.25 ng μl^{-1} (30 µl). The solution was then run through a pipette packed with silica gel and eluted with methylene chloride. Both methanol and methylene chloride eluates were collected in one vial for a combined volume of 500 µl. This step removed colored pigments from the solution. The combined eluate was evaporated under a gentle stream of nitrogen to ~30 µl.

Gas Chromatography

Extracts were analyzed on a Hewlett Packard 6890 series gas chromatograph (Agilent Technologies: Santa Clara CA.), equipped with a non-polar capillary column (DB-5, 30 m x 0.32 mm x 0.25 µm film; J&W Scientific, Folsom CA.). The GC injection port was set to 260° C. The column temperature was held at 40° C for 1 min, then increased to 250° C at 5° C min^{-1}. Helium was used as a carrier gas at 1 ml min^{-1}, and samples were injected in splitless mode with a purge time of 1 min.

Compounds were tentatively identified by matching mass spectra with those in the NIST Library mass spectral database. Identities of methoxypyrazines were confirmed by matching retention times and mass spectra with those of synthetic standards (2-isobutyl-, [IBMP]; 2-sec-butyl-, [SBMP]; and 2-isopropyl-3-methoxypyrazines, [IPMP]; Aldrich Chemical Co.) An external

standard of hippodamine was not available, but its identity was supported by previous identifications and published mass spectra of hippodamine from *H. convergens* (Tursch et al., 1974; King and Meinwald, 1996; Slogget et al., 2009; Kajita et al., 2010). The concentrations of hippodamine and methoxypyrazines were estimated using area comparisons to internal standards that possess similar chemical properties (sparteine and 2-methoxy-3-methylpyrazine respectively).

Results

All (n=640) beetles analyzed had an R' color value between 254.12' and 255' (x=254.64±0.87). All the variation in the color was due to the different contributions of G' and B' coordinates. There was a linear correlation between G' and B' (Fig. 13). The consistency of the R' value, and the positive linear correlation of G' and B' indicate that the average R'G'B' histogram value would account for all variation in visual color spectrum and could thus be a valid comparative descriptor of elytra color between beetles (preliminary work with a spectrophotometer identified no reflectance within the UV range). Beetle elytra coloration was normally distributed (Shapiro-Wilk: P=0.47) around a R'G'B' mean of 176.5'±13.2' (Fig. 14), with males slightly skewed towards the lighter, orange end of the red spectrum.

Females and male beetles possessing the lower and upper 25% R'G'B' histogram values were used for chemical analysis. This quantile delineation yielded 8 samples of 10 beetles for each of the four treatment types: "red" females (R'G'B'<167.6'), "orange" females (R'G'B'>185.3'), red males, and orange males. Those beetles possessing the middle 50% R'G'B' values were omitted from further analysis.

As reported by Kajita et al. (2010), convergine breaks down into multiple products on the GC (Fig. 15A,C). Using a diboron agent to reduce this *N*-oxide to the free-base, hippodamine, greatly reduced the number and size of peaks surrounding hippodamine (Fig. 15B). The reduction of convergine to hippodamine thus coalesced the alkaloids into one peak, allowing for more accurate quantitation. Red females contained significantly higher concentrations of alkaloids than orange females, red males, or orange males (Fig. 16). Red males had significantly more alkaloids than orange males, whereas orange females were not significantly different than either group of males.

The average *H. convergens* possessed 93.4 ng IPMP, 15.0 ng SBMP, and 29.4 ng IBMP. Males possessed significantly greater amounts of methoxypyrazines than females (ANOVA: F_{32}=22.04, P<0.0001). Methoxypyrazine concentration was not completely correlated with coloration, because the direction of correlation was opposite between the sexes (Fig. 15). The total methoxypyrazine concentration of red males was significantly higher than orange males (GLM LS means Tukey test: P=0.043) and orange females (Tukey test: P=0.002). Unlike their red male counterparts, however, red females had significantly less pyrazines than orange females (Tukey test: P=0.047). Red females had ~ twofold more alkaloids than the other treatment groups.

Combining sex and coloration groupings together revealed that methoxypyrazines concentration was negatively correlated with alkaloid concentration (Fig. 18; ANOVA regression: F_{32}=19.99, P=0.00019; R^2=0.48). Separately, all sex and coloration treatment groups shared similar negative correlations except for orange males (ANOVA regression: F_8=0.016, P=0.9; R^2=0.0026). Removing the single outlier possessing a uniquely high concentration of

methoxypyrazines (21.3 ng^{-1} mg) restored a highly significant negative correlation within this treatment group (ANOVA regression: F_7=67.86, P<0.00043).

Discussion

Conclusion

The coloration of *H. convergens* elytra ranges from a darker, saturated red to a lighter, more translucent yellow-orange. Beetles were normally distributed along this spectrum. Within each sex, the beetles on the red end of the spectrum possessed significantly higher concentrations of alkaloids than those on the orange end of the spectrum. There was a negative correlation between alkaloid concentration and methoxypyrazines concentration across all beetles, as well as within each sex/color treatment. Among males, those beetles on the red end of the spectrum possessed significantly higher concentrations of total methoxypyrazines than male beetles on the orange end of the spectrum. Among females, however, the opposite was true, with the redder females possessing the lowest concentration of total methoxypyrazines. Red females have a comparably much larger concentration of alklaloids than other treatment groups, and it would appear that the negative correlation between alkaloids and methoxypyrazines, supersedes the coupling of the color display and methoxypyrazines.

Honest visual signals of toxicity

Red coloration, especially red-black contrast, is a well-established visual signal of toxicity. A strong coupling between an indicator trait and the defensive trait is integral to the evolution of aposematism, yet there is little empirical evidence of a positive linear correlation between the two (Grill, 1999). Here we have shown that those beetles with more saturated red coloration possessed significantly more defensive alkaloids than their orange-colored counterparts. We

therefore suggest that *H. convergens* visual aposematic signals are quantitatively honest signals of toxicity.

Some theories on aposematism predict a negative correlation between conspicuous aposematic advertisements and toxicity, on the assumption that conspicuous signals are costly because they attract predator attention (Leimar et al., 1986; Speed and Ruxton, 2005). Although negative correlations can exist between signal and defensive capabilities, the reasoning in this theory is based on the assumption that conspicuous signals are always handicaps because they attract the attention of predators.

In many cases, predators are most averse to the brightest, largest, most novel, or most conspicuous prey item (Marples and Roper, 1996). An animal's stimulus-response is primarily based on the discrimination of positive (S+) and negative (S-) stimuli along the same stimulus dimension (size: tiny to big; color: dull to bright). There is a certain property on the higher range of the linear dimension of a stimulus (size: biggest; color: brightest) that is by its very nature more excitatory and thus warrants larger impressions on perception and increases the efficiency of learning (Spence, 1937). This asymmetrical, stimulus-generalization gradient is the foundation of peak-shift theory (Gamberale and Tullberg, 1996) that currently serves as the governing principle supporting aposematism as an evolutionarily stable strategy (Coppinger, 1969, 1970; Leimar et al., 1986; Gamberale and Tullberg, 1996).

Peak-shift theory would assume that brighter red *H. convergens* elytra would present a stronger visual signal of toxicity, whereas the less apparent yellow-orange elytra would serve as a weaker signal more likely to fail at repelling predators. The redder elytra are fairly opaque, and when they lay over the abdomen of the beetle they maintain their red coloration and bold

black spots. The more orange elytra are translucent, and when they lay over the beetle they allow some of the black pigmentation of the abdomen to show through. This has the effect of darkening the orange color of the elytra to a dull reddish brown and reducing the contrast between the black spots and the colored background. Reducing the apparency of black spots on elytra would weaken their visual signal because, besides red color alone, the high contrast pattern on elytra reduces predation (Dolenska et al., 2009).

The coloration of the coccinellid elytra is derived from carotenoid-based pigmentation (Britton et al., 1977). Carotenoids are acquired from their diet (aphids contain carotenoids), and thus their display as pigment on the insect cuticle could function as honest signals of health, foraging abilities, and immunocompetence (Möller et al., 2000). *Harmonia axyridis* larvae that were fed on a sub-optimal diet of pollen developed into paler adults than those larvae fed on aphids (Grill and Moore, 1998). Carotenoid-based coloration is often used as a sexual signal, especially among birds and fish, and may have similar functions among insects, though variation in insect coloration and its relation to mate choice has rarely been examined. Coccinellids, however, may be unable to perceive the red wavelengths. Electroretinogram responses from the compound eyes of *C. septempunctata* produced a spectral sensitivity curve with two peaks: one near the longwave UV band (360-380 nm) and the other in a green band (510-530 nm; Lin and Wu, 1992). Additional tests indicate that *C. septempunctata* also likely posses a third photoreceptor expressing some sensitivity to wavelengths between the UV and green band, possibly blue, but this would leave very little sensitivity to colors at the red end of the spectrum. Therefore, if coloration were involved in mate choice, *H. convergens* would likely be responding to the degree of contrast between the red and black on conspecifics' elytra. Their ability to

perceive elytral pattern is supported by evidence that migrant coccinellids orient toward visually contrasting features at a variety of spatial scales (Hodek et al., 1993; Nalepa et al., 2005). There also is evidence that color pattern may influence female mate choice, at least among the color pattern extremes between melanic and non-melanic *H. axyridis* and *Adalia bipunctata* (Majerus et al., 1982; Obata, 1988; Osawa and Nishida, 1992). It may be beneficial for females to mate with highly protected males, because there is evidence in one coccinellid species that males' endogenous alkaloids contribute to the chemical defenses of females' eggs (Camarano et al., 2009).

The coupling of visual and olfactory aposematic signals.

Among males there is a positive correlation between the intensity of red coloration and methoxypyrazine concentration. This pairing of the two displays suggests that aposematism may follow a "redundant signal" content-based hypotheses among male *H. convergens*. This hypothesis predicts positive correlations between different signals if those signals all provide the same information and function best to reduce predation when presented in combination (Hebets and Papaj, 2005). Although visual and olfactory coccinellid signals may best reduce predation when presented in combination (Rowe, 1999; Siddall and Marples, 2008), they do not necessarily provide the same information as to a beetle's toxicity. It is true that red males as a group have more methoxypyrazines and alkaloids than orange males, but within these two treatment groups there is significant negative correlation between alkaloids and methoxypyrazines.

When methoxypyrazines are expressed with a prey item, they are not always aversive to predators (Marples and Roper, 1996), but when presented simultaneously with warning colors and/or aggregations, avoidance is induced or significantly enhanced in predators (Rowe, 1999;

Riipi et al., 2001; Siddall and Marples 2008). In *H. convergens* the production of methoxypyrazines has the added benefit of functioning as an aggregation pheromone (Wheeler and Cardé, 2013). Thus by increasing production of methoxypyrazines, avoidance learning efficiency is improved both through the combined interaction of warning odor with warning coloration and aggregations with warning coloration. It may be that these aposematic interactions select for methoxypyrazines to be positively correlated with the intensity of red coloration, but that the energetic requirements or resource limitations of producing high concentrations of both methoxypyrazines and alkaloids conflict (respectively the "increased learning and memory" and the "multitasking" inter-signal interaction hypotheses of Hebets and Papaj [2005]).

Although the production costs of hippodamine have not been quantified in *H. convergens*, it is endogenous in origin and is likely costly to produce, as has been demonstrated in *Coccinella septempunctata* (Holloway et al., 1991), *A. bipunctata* (de Jong et el., 1991), and other beetles (Rowell-Rahier and Pasteels, 1986). Any increase in factors that improve the efficiency of predator avoidance learning is theorized to select for reduced investment in actual chemical defenses (Skelhorn and Ruxton, 2008). Higher methoxypyrazine concentrations and brighter red aposematic coloration would improve the efficacy of a coccinellid's warning signal, thus speeding up predator avoidance learning and reducing the chance that the alkaloid defenses will be utilized. As a result, an aposematic prey which is highly efficient at signaling will benefit less from investing in costly defenses. This might explain why, although the different male coloration groups have different starting average methoxypyrazine concentrations, the rate at which methoxypyrazines concentrations increase as alkaloid concentrations decrease is

the same (Fig. 18). A similar negative correlation carries over for females as well. The correlations between methoxypyrazines and alkaloids within red females and red males share similar slopes, as can be seen from the highly-fitted trendline running through both series (though methoxypyrazine concentrations plateau at alkaloid concentrations above ~1450 ng mg^{1} alkaloids).

Why do females have more alkaloids than males?

Red females were found to possess significantly more alkaloids than red males. Although saturation of red coloration is correlated with alkaloid concentration in both sexes, some selective pressures must explain this trend in females. *Adalia bipunctata* females also produce significantly higher concentrations than males of their defensive alkaloid, adaline (Holloway et al., 1993). Holloway et al. (1993) suggest that different constraints on the production of alkaloids operate among the sexes of *A. bipunctata*. Due to the costs associated with egg laying, females and males have different sets of energetic and nutritional constraints. Additionally, females impart a fraction of their total alkaloid content to their eggs (Pasteels, 2007). Among *Epilachna paenulata*, the eggs even contain higher concentrations of alkaloids than the adults (Camarano et al., 2006). Only one or a few of these bright yellow eggs would be eaten by a predator before the alkaloid would likely repel the predator and ensure the survival of the remaining eggs. Therefore, the differing roles of males and females in reproduction likely results in partitioning of available energy and resources in different ways among the various important fitness functions.

Future directions

For quantification of methoxypyrazines to be possible with the methodology of this study, beetles had to be extracted in groups of 10. This procedure hides individual variation and may

reduce the differences between treatments. This methodology also necessitated that the continuous coloration variables be grouped into categorical variables. Future experiments that utilize static headspace sampling techniques (Cudjoe et al., 2005) or solid-phase microextraction in multidimensional gas chromatography (Cai et al., 2007; Schmarr et al. 2010) could further examine the extent of the inter-signal relationship of methoxypyrazines with coloration and alkaloid toxicity.

Any negative correlation found between aggregative behavior and the aposematic signals measured in this study may reflect how beetles compensate for their reduced ability to produce warning signals during harsh overwintering conditions by increasing their conspicuousness through the formation of an aggregation. Alternatively, the defensive benefits of aggregating may reduce the selective benefits of producing the actual distasteful toxins such signals advertise (Skelhorn and Ruxton, 2008). If so, automimicry may be present in the aggregation, as indicated by only some individuals in the aggregation being highly defended. Additionally, because there is evidence that males contribute to the chemical defenses of their offspring (Camarano et al., 2009), it would follow that females would benefit by mating with more highly defended males. Additionally, males should favor mating with the reddest females because these possess the highest alkaloid content, a factor in egg protection. Therefore, another direction to pursue would be the roles that the warning signals of color and odor may play in mate selection.

Literature Cited

Britton, G., Lockley, W. J. S., Harriman, G. A. and Goodwin, T. W. 1977. Pigmentaiton of ladybird beetle *Coccinella septempunctata* by carotenoids not of plant origin. *Nature*. 266:49-50.

Cai, L. S., Koziel, J. A., and O'Neal, M. E. 2007. Determination of characteristic odorants from *Harmonia axyridis* beetles using in vivo solid-phase microextraction and multidimensional gas chromatography-mass spectrometry-olfactometry. *Journal of Chromatography A*. 1147:66-78.

Camarano, S., González, A., and Rossini, C. 2006. Chemical defense of the ladybird beetle *Epilachna paenulata*. *Chemoecology*. 16:17-184.

Camarano, S., González, A., and Rossini, C. 2009. Biparental endowment of endogenous defensive alkaloids in *Epilachna paenulata*. *Journal of Chemical Ecology*. 35:1-7.

Coppinger, R.P. 1969. The effect of experience and novelty on avian feeding behavior with reference to the evolution of warning coloration in butterflies - Part I. *The American Naturalist*. 104:323-335.

Coppinger, R.P. 1970. The effect of experience and novelty on avian feeding behavior with reference to the evolution of warning coloration in butterflies - Part II. *The American Naturalist*. 106:48-59.

Cudjoe, E., Wiederkehr, T. B., and Brindle, I. D. 2005. Headspace gas chromatography-mass spectrometry: a fast approach to the identification and determination of 2-alkyl-3-methoxypyrazine pheromones in ladybugs. *Analyst.* 130:152-155.

Dolenská, M., Nedvěd, O., Veselý, Tesařová, M., and Ruchs, R. 2009. What constitutes optical warning signals of ladybirds (Coleoptera: Coccinellidae) towards bird predators: colour, pattern or general look? *Biological Journal of the Linnean Society*. 98:234-242.

Gamberale, G. and Tullberg, B. S. 1996. Evidence for a peak-shift in predator generalization among aposematic prey. *Proceedings: Biological Sciences*. 263:1329-1334.

Gohli, J. and Hogstedt, G. 2010. Reliability in aposematic signaling: thoughts on evolution and aposematic life. *Communicative and Integrative Biology.* 3:9-11.

Grill, C. P. 1999. Development of colour in an aposematic ladybird beetle: the role of environmental conditions. *Evolutionary Ecology Research*. 1:651-662.

Hebets, E. A. and Papaj, D. R. 2005. Complex signal function: developing a framework of testable hypotheses. *Behavioral Ecology and Sociobiology.* 57:197-214.

Hodek, I., and Honêk, A. 1996. *Ecology of Coccinellidae.* Boston: Kluwer Academic.

Hodek, I., Iperti, G., and Hodkova, M. 1993. Long-distance flights in Coccinellidae (Coleoptera). *European Journal of Entomology*. 90:403-414.

Holloway, G. J., de Jong, P. W., Brakefield, P. M., and de Vos, H. 1991. Chemical defence in ladybird beetles (Coccinellidae). 1. Distribution of coccinelline and individual variation in defence in 7-spot ladybirds (*Coccinella septempunctata*). *Chemoecology.* 2:7-14.

Holloway, G. J., de jong, P. W., and Ottenheim, M. 1993. The genetics and cost of chemical defense in the 2-spot ladybird (*Adalia bipunctata* L). *Evolution.* 47:1229-1239.

de Jong, P. W., Holloway, G. J., Brakefield, P. M., and de Vos, H. 1991. Chemical defence in laybird beetles (Coccinellidae). 2. Amount of reflex fluid, the alkaloid adaline and individual variation in defence in 2-spot ladybirds (*Adalia bipunctata*). *Chemoecology*. 2:15-19.

Kajita, Y., Obrycki, J. J., Sloggett, J. J., and Haynes, K. F. 2010. Intraspecific alkaloid variation in ladybird eggs and its effects on con- and hetero-specific intraguild predators. *Oecologia*. 163:313-322.

King, A. G. and Meinwald, J. 1996. Review of the defensive chemistry of coccinellids. *Chemical Reviews*. 96:1105-1122.

Kokatla, H. P, Thomson, P. F., Bae, S., Doddi, V. R., and Lakshman, M. K. 2011. Reduction of amine *N*-oxides by diboron reagents. *Journal of Organic Chemistry*. 76: 7842-7848.

Leimar, O., Enquist, M., Sillen-Tullberg, B. 1986. Evolutionary stability of aposematic coloration and prey unprofitability: A theoretical analysis. *The American Naturalist*. 128:469-490.

Lin J. -T. and Wu, C. –Y. 1992. A comparative study on the color vision of four coleopteran insects. *Bulletin of the Institute of Zoology Academia Sinica (Taipei)*. 31:81-88.

Majerus, M. E. N. and Kearns, P. 1989. Ladybirds: Naturalist Handbooks 10. Richmond Publishing Co Ltd., Slough, England.

Majerus, M. E. N., Odonald, P., and Weir, J. 1982. Female mating preference is genetic. *Nature*. 300:521-523.

Marples, M. E. N. and Roper, T. J. 1996. Effects of novel colour and smell in the response of naïve chicks towards food and water. *Animal Behaviour*. 51:1417-1424.

Marples, M. E. N., Vanveelen, W., and Brakefield, P. M. 1994. The relative importance of colour, taste and smell in the protection of an aposematic insect *Coccinella septempunctata*. *Animal Behaviour.* 48:967-974.

Möller, A. P., Biard, C., Blount, J. D., Houston, D. C., Ninni, P., Saino, N., and Surai, P. F. 2000. Carotenoid-dependent signals: Indicators of foraging efficiency immunocompetence or detoxification ability? *Avian and Poultry Biology Reviews*. 11:137-159.

Obata, S. 1988. Mating refusal and its significance in females of the ladybird beetle, *Harmonia axyridis*. *Physiological Entomology.* 13:193-199.

Osawa, N., and Nishida, T. 1992. Seasonal variation in elytral color polymorphism in *Harmonia axyridis* (the ladybird beetle): the role of nonrandom mating. *Heredity*. 69:297-307.

Pasteels, J. M. 2007. Chemical defence, offence and alliance in ants-aphids-ladybirds relationships. *Poplulaiton Ecology*. 49:5-14.

Riipi, M., Alatalo, R. V., Lindstrom, L., and Mappes, J. 2001. Multiple benefits of gregariousness cover detectability costs in aposematic aggregations. *Nature.* 413:512-514.

Rowell-Rahier, M. and Pasteels, J. M. 1986. Economics of chemical defense in Chrysomelinae. *Journal of Chemical Ecology*. 12:189-1203.

Rowe, C. 1999. Receiver psychology and the evolution of multicomponent signals. *Animal Behaviour*. 58:921-931.

Rowe, C. and Guilford, T. 1999. The evolution of multimodal warning displays. *Evolutionary Ecology*. 13:655-671.

Schmarr, H-G., Ganβ, S., Koschinski, S., Fischer, U. Riehle, C., Kinnart, J., Potouridis, T., and Kutyrev, M. 2010. Pitfalls encountered during quantitiative determination of 3-alkyl-2-methoxypyrazines in grape must and wine using gas chromatogorphay-mass spectrometry with stable isotope dilution analysis. Comprehensive two-dimensional gas chromatogorpahy-mass spectrometery and on-line liquid chromatogorphy-multidimensional gas chromatogrophy-mass spectromeetry as potential loopholes. *Journal of Chromatography A*. 1217:6769-6777.

Siddall, E. C. and Marples N. M. 2008. Better to be bimodal: the interaction of color and odor on learning and memory. *Behavioral Ecology.* 19:425-432.

Skelhorn, J. and Ruxton, G. D. 2008. Ecological factors influencing the evolution of insects' chemical defenses. *Behavioral Ecology.* 19:46-153.

Sloggett, J. J., Obrycki, J. J., and Haynes, K. F. 2009. Identification and quantification of predation: novel use of gas chromatography-mass spectrometric analysis of prey alkaloid markers. *Functional Ecology*. 23:416-426.

Speed, M. P. and Ruxton, G. D. 2005. Aposematism: What should our starting point be? *Proceedings: Biological Sciences*. 272:431-438.

Spence, K. W. 1937. The differential response in animals to stimuli varying within a single dimension. *Psychological Review*. 44:430-444.

Tursch, B., Daloze, D., Braekman, J. C., Hootele, C., Cravador, A., Losman, D., and Karlsson, R. 1974. Chemical ecology of arthropods 9: structure and absolute configuration of hippodamine and convergine, 2 novel alkaloids from American ladybug *Hippodamia convergens* (Coleoptera-Coccinellidae). *Tetrahedron Letters.* 15:409-412.

Vogler, A. P. and Kelley, K. C. 1998. Covariation of defensive traits in tiger beetles (genus Cicindela): a phylogenetic approach using mtDNA. *Evolution*. 52:529-538.

Wheeler, C. A., and Cardé, R. T. 2013. Defensive allomones funciton as aggregation pheromones in diapausing ladybird beetles, Hippodamia convergens. *Journal of Chemical Ecology.* 39:723-732.

Tables and Figures

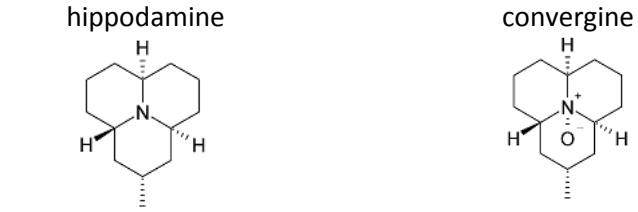

Figure 13 The chemical structures and diagnostic ions of *Hippodamia convergens* endogenous azaphenalene alkaloids: Pyrido[2,1,6-de]quinolizine, dodecahydro-2-methyl-10-oxide, (2α,3aS,6aα,9aS,10α)- and its *N*-oxide chiral form, 10-oxide, (2α,3aβ,6aα,9aβ)- respectively.

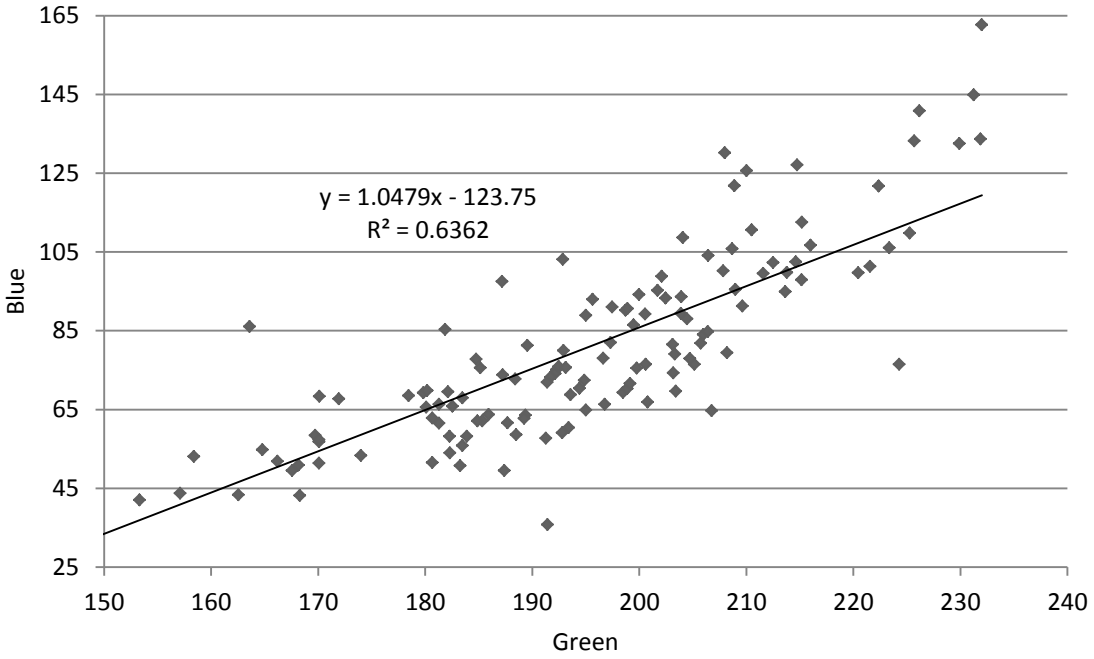

Figure 14 Correlation between G' (green) and B' (blue) R'G'B' histogram values from the "red" portion of 130 *Hippodamia convergens* elytra. Linear trend-line equation and R^2 value displayed within the graph.

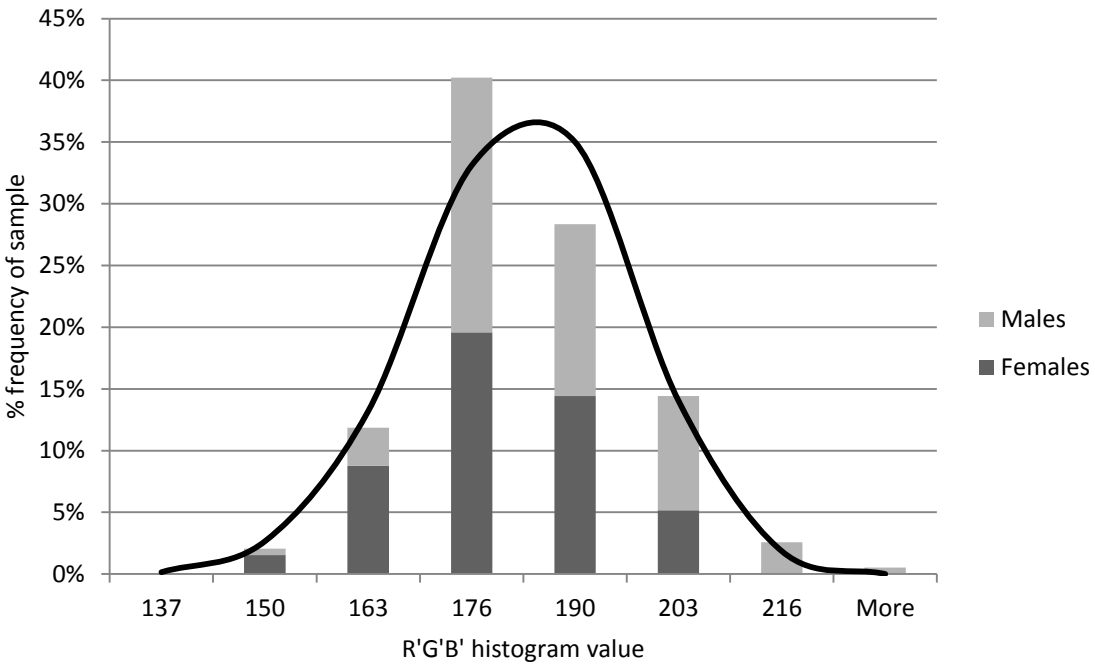

Figure 15 Frequency histogram of the average R'G'B' values from the "red" portion of 130 individual *Hippodamia convergens* elytra. Solid line shows a normal distribution.

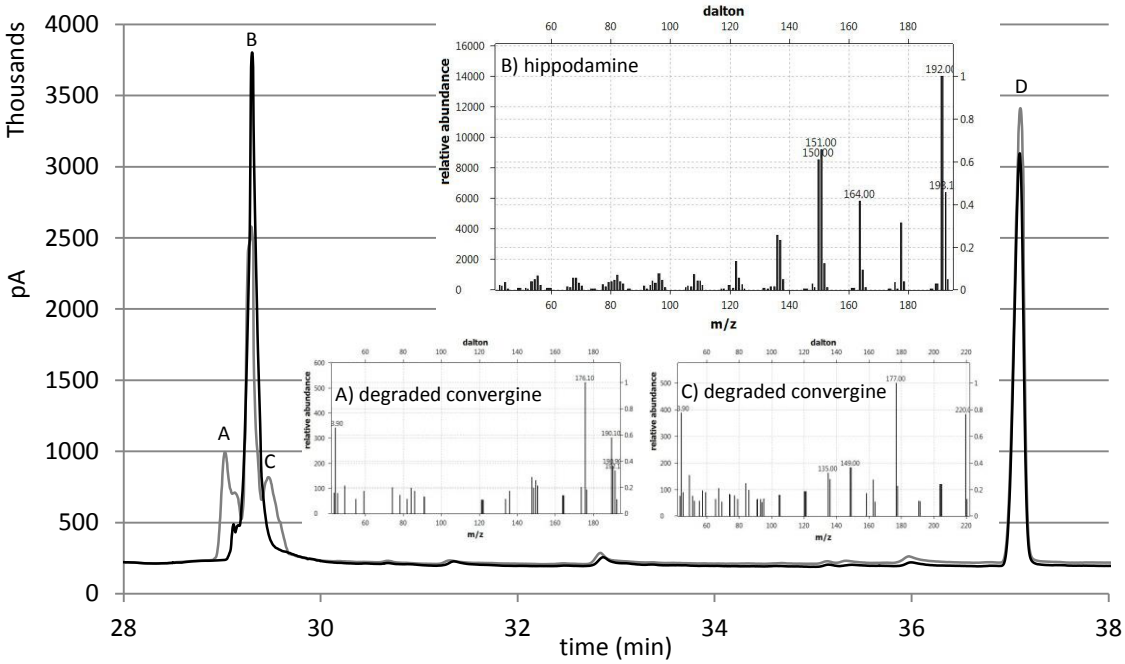

Figure 16 The portion of the GC trace obtained from a whole *Hippodamia convergens* extract containing the naturally-occurring alkaloids (A, B, and C) and an internal standard of synthetic sparteine (D). Histogram insets depict the mass spectra and identification of select chromatographic peaks. The grey trace depicts the original extract injection, with the hippodamine, B, peak at ~29.3 min, and the degraded convergine products, A and C, surrounding it. The black trace shows the same extract after reduction of the N-oxide, convergine. The small peak on the leading edge of the hippodamine is typical of most reduced extract samples, and represents a small amount of convergine product that was not successfully reduced to hippodamine. Its contribution appears minimal and was included into the quantification of the single hippodamine peak.

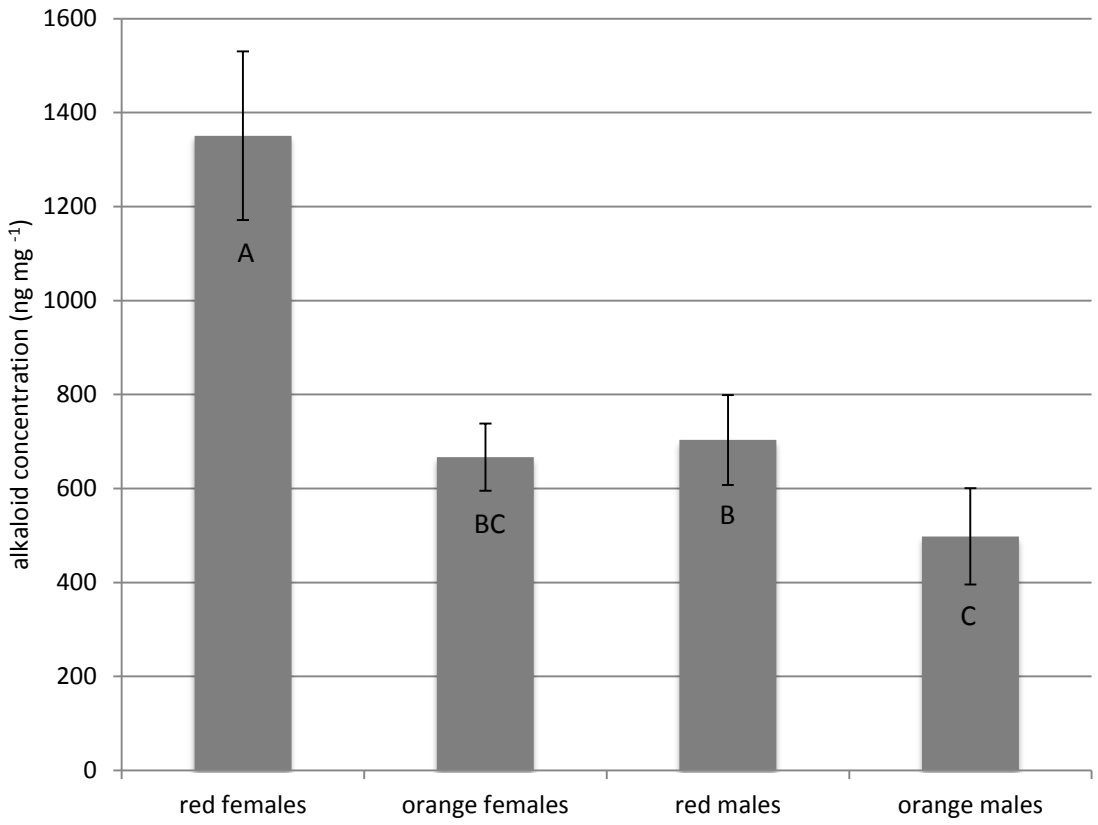

Figure 17 Bar graph depicting the difference in alkaloid concentrations between the coloration categories of male and female *Hippodamia convergens*. Alkaloid concentration reported as ng of hippodamine per mg of beetle. Beetles with elytra possessing an average R'G'B' histogram < 167.6' qualify as "red," and those > 185.3' qualify as "orange." Letters indicate significant differences (GLM LS means Tukey test, $P < 0.0125$). N=8, each replicate represents 10 beetles.

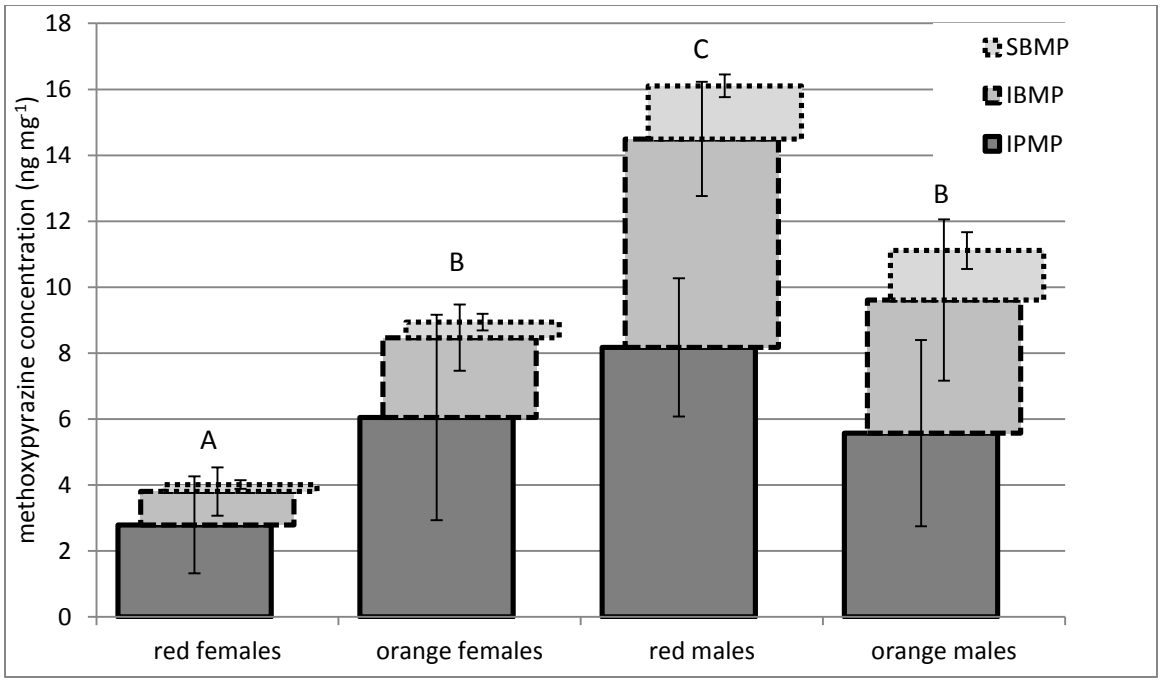

Figure 18 Average concentration of each methoxypyrazine extracted from *Hippodamia convergens* females and males of two different coloration groups (red vs orange): SBMP= 2-sec-butyl-3-; IBMP = 2-Isobutyl-3-; and IPMP = 2-isopropyl-3-methoxypyrazine. (GLM LS means Tukey test, $P<0.05$). *N*=8, each replicate contains calculations from a sample of 10 beetles.

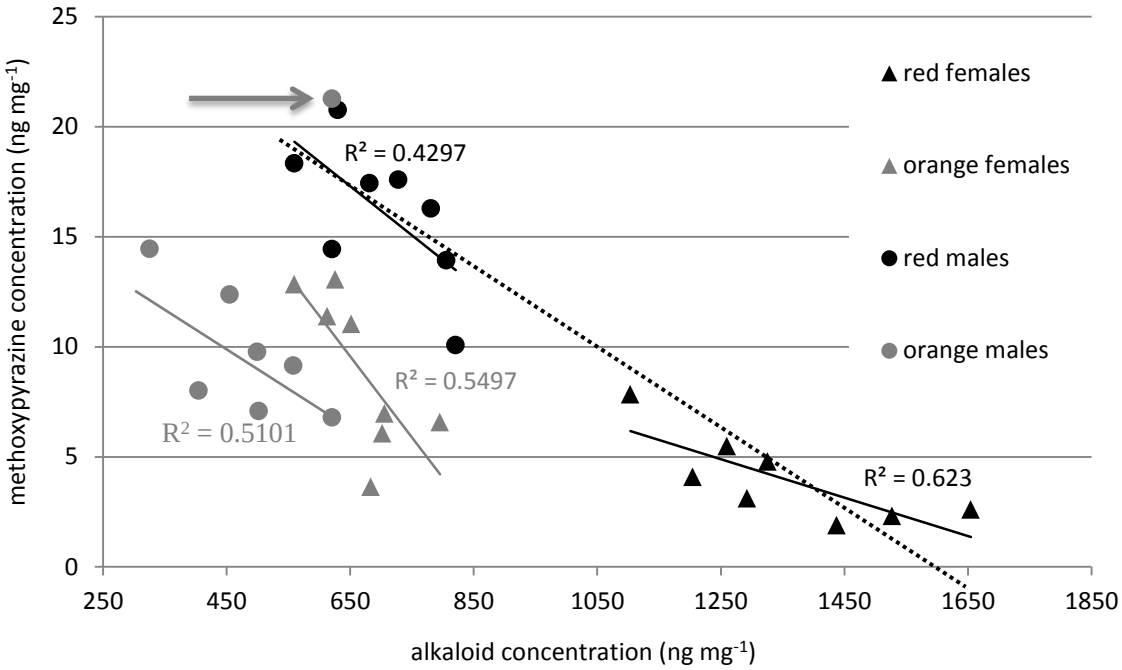

Figure 19 Correlation between the total methoxypyrazine concentration and the alkaloid concentration of *Hippodamia convergens* extracts. Symbols depict each sex and coloration treatment group. Arrow indicates potential outlier within the orange males treatment group. Linear trendlines are drawn as solid lines through each series (trendline for orange male's omits outlier). The dotted line depicts the linear trendline for both red sexes pooled together (R^2=0.89).

Concluding Remarks

The experiments reported in this dissertation demonstrate that chemical communication is, at least in part, responsible for the formation and persistence of overwintering aggregations of *Hippodamia convergens*. Individually, these semiochemicals influence the orientation of beetles in such a way as to lead to the formation of at least temporary aggregations. When both of these seimochemicals are used together, however, an aggregation persists. Both of the semiochemicals involved have supplemental functions that likely facilitated their evolution as aggregation signals. One of these semiochemicals is tricosane, a hydrocarbon in the footprints of walking beetles. This hydrocarbon likely persists on the surface, labeling microsites as optimal communal hibernacula for subsequently arriving migrants. The other aggregation pheromone's (2-isobutyl-3-methoxypyrazine [IBMP]) supplemental function is as an aposematic odor, and it varied predictably with toxicity and another aposematic signal, red coloration.

The first chapter of this dissertation outlines the life history of *H. convergens*, with special attention to their overwintering behavior. I explain that while the mass arrival of *H. convergens* to communal hibernacula is a well-known phenomenon, neither the mechanisms of this behavior nor the adaptive function of the aggregation were clearly defined. I hypothesized that chemical communication may be involved in the formation and cohesion of these aggregations, and that the determination of these semiochemicals would help further our understanding of the evolution of aposematism and aggregation.

The second chapter reports an experiment demonstrating that *H. convergens* aggregate to the odor of conspecifics. A blend of methoxypyrazines, but particularly IBMP (and to a lesser extent, 2-sec-butyl-methoxypyrazine [SBMP]), were identified as the odor compounds

responsible for this aggregative behavior. Chapter three reports on an experiment demonstrating that conspecific hydrocarbons interact with the methoxypyrazine pheromone to complete the *H. convergens* aggregation signal. Tricosane is the primary hydrocarbon found within the blend of cuticular hydrocarbons that influences *H. convergens* orientation. The orientation response of diapausing females to this hydrocarbon differed from that of non-diapausing females. This context-specific behavioral variation highlights the supplemental functions of *H. convergens* cuticular hydrocarbons as oviposition deterrents (reviewed in Seagraves, 2009; in *H. convergens* Michaud and Jyoti, 2007), and species/gender-specific cues (Hemptinne et al. 1998; Omkar et al. 2004).

The fourth chapter of this dissertation transitions to an analysis of the supplemental aposematic functions of methoxypyrazines. I conducted this analysis by quantifying the toxicity, and the olfactory and visual aposematic signals of *H. convergens.* I discovered that overall, methoxypyrazines are negatively correlated with toxicity, whereas color is positively correlated with toxicity. Interestingly, among all but the most toxic females, color appeared to be positively correlated with methoxypyrazines. These apparent conflicting correlations are explained by considering complex signal theory. There are several mechanisms by which the energy or dietary requirements of producing alkaloid toxins might limit the production of methoxypyrazines (Hebets and Papaj, 2005; Skelhorn and Ruxton, 2008). Additionally, the interaction between color and methoxypyrazines odor in predator avoidance learning (Siddall and Marples, 2008) may strongly couple these two traits when production is not limited.

This dissertation provides evidence that *H. convergens,* and potentially other coccinellids, respond to a series of multi-modal cues and signals across multiple spatial scales

when they are migrating to communal hibernacula. The following is my proposed summation of all the mechanisms involved in the arrival of migrants to these hibernacula. Initially, the temperature flight ceiling, prevailing westerly winds (Hagen, 1962), and hypsotactic orientation (movement toward prominent, isolated objects on the horizon) lead migrants to mountain ranges. From there, hypsotaxis towards clumps of rocks or isolated trees, and positive phototaxis to sunny clearings further concentrates the migrant populations to particular overwintering macrosites (Hodek and Honêk, 1996; Nalepa et al., 2005). At this point, beetles switch from flying to walking and can orient to hibernacula possessing abiotic conditions favorable for overwintering. Orientation mechanisms such as positive geotaxis, thigmotaxis, hygrotaxis, and temperature-regulated phototaxis would facilitate the arrival of individuals to these microsites (Hodek and Honêk, 1996; Copp, 1983). If these were the only cues *H. convergens* responded to, aggregations at these sites would be an incidental result of many individuals shared responses to those particular abiotic conditions. Orientation within this heterogeneous landscape of favorable abiotic stimuli, however, is likely overlaid with the persistent hydrocarbons left behind by previous generations of migrants. *Hippodamia convergens* are aposematic and would benefit from aggregating (Gamberale and Tullberg, 1998; Riipi et al., 2001), thus conspecific attraction has evolved. These hydrocarbon footprint residues likely serve to indicate the presence of conspecifics, or at least mark hibernacula potentially possessing favorable overwintering conditions. Individual beetle's preference for conspecific hydrocarbon deposits are amplified by positive feedback (Dussutour et al., 2005; Jeanson et al., 2005) and the migrant population is further concentrated to those select microsites that contain this chemical signature. As individuals collect at these microsites, the methoxypyrazines they passively release also concentrate and likely serve as a more effective aposematic signal

(Kaufmann, 1966; Aldrich and Blum, 1978; Moore et al., 1990), and likewise a stronger aggregation signal for incoming conspecifics. Subsequently arriving *H. convergens* will be able to orient toward these communal hibernacula more effectively than earlier arriving migrants, and aggregation will grow exponentially. The formation of these overwintering aggregations will potentiate aposematic signals of color (Hagen, 1962; Majerus, 1994) and odor (Kaufmann, 1966; Aldrich and Blum, 1978; Moore et al., 1990), increasing individuals' chances of survival, and selection for the reformation of aggregations at these same hibernacula in subsequent years.

Literature Cited

Aldrich, J. R. and Blum, M. S. 1978. Aposematic aggregation of a bug (Hemiptera: Coreidae): defensive display and formation of aggregations. *Biotropica.* 10:58-61.

Copp, N. H. 1983. Temperature-dependent behaviours and cluster formation by aggregating ladybird beetles. *Animal Behaviour.* 31:424-430.

Dussutour, A., Deneuboug, J., and Fourcassie, V. 2005. Amplification of individual preferences in a social context: The case of wall-following in ants. *Proceedings of the Royal Society of Biological Sciences.* 272:705-714.

Gamberale, G. and Tullberg, B. S. 1998. Aposematism and gregariousness: the combined effect of group size and colouration on signal repellence. *Proceedings of the Royal Society of London B Biological Sciences.* 265:889-894.

Hagen, K. S. 1962. Biology and ecology of predaceous Coccinellidae. *Annual Review of Entomology* 7:289-326.

Hebets, E. A. and Papaj, D. R. 2005. Complex signal function: developing a framework of testable hypotheses. *Behavioral Ecology and Sociobiology.* 57:197-214.

Hemptinne, J. L., Lognay G., and Dixon A. F. G. 1998. Mate recognition in the two-spot ladybird beetle, *Adalia bipunctata*: role of chemical and behavioural cues. *Journal of Insect Physiology*. 44:1163-1171.

Hodek, I. and Honěk, A. 1996. Ecology of Coccinellidae. Kluwer Academic Publishers, Boston.

Jeanson, R., Rivault, C., Deneubourg, J-L., Blanco, S., Fournier, R., Jost, C., and Theraulaz, G. 2005. Self-organized aggregation in cockroaches. *Animal Behaviour*. 69:169-180.

Kaufmann, T. 1966. Observations on some factors which influence aggregation by *Blaps sulcata* (Coleoptera - Tenebrionidae) in Israel. *Annals of the Entomological Society of America.* 59:660-664.

Majerus, M. E. N. 1994. Ladybirds. Harper Collins, London.

Michaud, J. P., and Jyoti, J. L. 2007. Repellency of conspecific and heterospecific larval residues to *Hippodamia convergens* (Coleoptera: Coccinellidae) ovipositing on sorghum plants. *European Journal of Entomlogy.* 104:399-405.

Moore, B. P., Brown, V. W., and Rothschild, M. 1990. Methylalkylpyrazines in aposematic insects, their hostplants and mimics. *Chemoecology* 1:43-51.

Nalepa, C. A., Kennedy, G. G., and Brownie, C. 2005. Role of visual contrast in the alighting behavior of *Harmonia axyridis* (Coleoptera: Coccinellidae) at overwintering sites. *Environmental Entomology.* 34:425-431.

Omkar, Pervez, A., and Gupta, A. K. 2004. Role of surface chemicals in egg cannibalism and intraguild predation by neonates of two aphidophagous ladybirds, *Propylea dissecta* and *Coccinella transversalis*. *Journal of Applied Entomology.* 128: 691-695.

Riipi, M., Alatalo, R. V., Lindstrom, L., and Mappes, J. 2001. Multiple benefits of gregariousness cover detectability costs in aposematic aggregations. *Nature.* 413:512-514.

Seagraves, M. P. 2009. Lady beetle oviposition behavior in response to the trophic environment. *Biological Control.* 51:313-322.

Siddall, E. C. and Marples N. M. 2008. Better to be bimodal: the interaction of color and odor on learning and memory. *Behavioral Ecology.* 19:425-432.

Skelhorn, J. and Ruxton, G. D. 2008. Ecological factors influencing the evolution of insects' chemical defenses. *Behavioral Ecology.* 19:46-153.