



# Carabidae Semiochemistry: Current and Future Directions

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## Abstract

Ground beetles (Carabidae) are recognized for their diverse, chemically-mediated defensive behaviors. Produced using a pair of pygidial glands, over 250 chemical constituents have been characterized across the family thus far, many of which are considered allomones. Over the past century, our knowledge of Carabidae exocrine chemistry has increased substantially, yet the role of these defensive compounds in mediating behavior other than repelling predators is largely unknown. It is also unclear whether non-defensive compounds produced by ground beetles mediate conspecific and heterospecific interactions, such as sex-aggregation pheromones or kairomones, respectively. Here we review the current state of non-exocrine Carabidae semiochemistry and behavioral research, discuss the importance of semiochemical research including but not limited to allomones, and describe next-generation methods for elucidating the underlying genetics and evolution of chemically-mediated behavior.

**Keywords** Carabidae · Chemical ecology · Allomones · Entomology · Semiochemistry · Transcriptomics · Phylogenetics

## Introduction

Ground beetles (Carabidae) have long captured the attention of evolutionary biologists and chemical ecologists due to their great diversity and array of chemical defensive strategies (Darwin 1846; Eisner 1958). Since Thomas Eisner's pioneering studies on bombardier beetles, knowledge of carabid defensive chemistry has grown tremendously, with over 250 distinct chemical classes currently described (Lečić et al. 2014). Despite the importance of non-allomonic semiochemicals in mediating interactions between and within species, studies on behaviorally-active compounds for any of the over 40,000 species of carabid remain absent (Arndt et al. 2005). Given that many of these compounds are important, if not crucial, to regulating behavior in insects (e.g. sex-aggregation pheromones), understanding their biosynthesis and diversity could provide new insights into the evolution of one of the animal kingdom's most biodiverse families. We review the current state of non-allomonic semiochemistry

and behavioral research in Carabidae and discuss new methods for studying the underlying genetics and evolution of biosynthetic pathways responsible for synthesis of these semiochemicals.

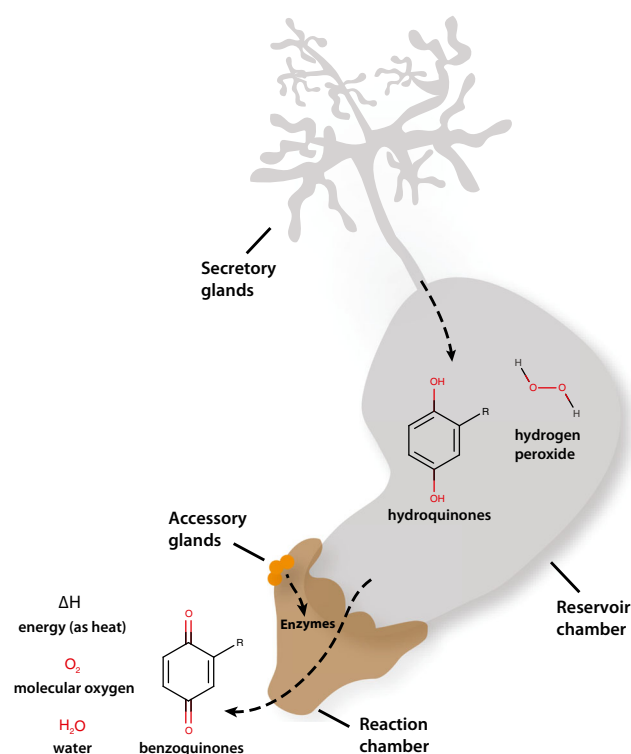
## Carabidae Semiochemistry

**Pygidial Gland Form and Function** Pygidial glands are located posterodorsally within the abdomens of all adephagans, which include Carabidae (Beutel and Haas 1996; Dettner 1985; Forsyth 1968, 1970, 1972). Each independent glandular system is composed of one set of multi-lobed secretory glands, which are connected to reservoir-pump chambers via long collecting ducts (Forsyth 1972; Di Giglio et al. 2011). Defensive chemicals are thought to be synthesized in the multi-lobed secretory glands, which funnel into the collecting duct, and ultimately collect in the reservoir chambers (Attygalle et al. 2006; Will et al. 2000). When a beetle is attacked, the pygidial glands are discharged by oozing the contents out of the tip of the abdomen, deterring the threat (Eisner et al. 1976, 2006). Within the Brachininae and Paussinae, however, an additional gross anatomical structure allows for the aiming and spraying of allomones at a target with great accuracy (Eisner and Aneshansley 1999). In these taxa, colloquially known as bombardier beetles, a highly-

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sclerotized, conical reaction chamber attaches to the posterior end of the reservoir and opens to the abdominal tip (Di Giulio et al. 2015; Eisner et al. 2000). Situated within the reaction chamber is a one-way valve that physically separates the contents of the reservoir chamber from the inside of the reaction chamber (Arndt et al. 2015). Accessory glands associated with the reaction chamber are thought to synthesize oxidative enzymes. When the valve is opened, hydroquinones and hydrogen peroxide rush into the reaction chamber, the hydroquinones are ultimately oxidized to p-benzoquinones, and the pressure build up ejects the mixture of newly oxidized quinones out of the abdomen at temperatures of up to 100 °C at a rate of 341 to nearly 1000 pulses per second (Fig. 1) (Aneshansley et al. 1969; Arndt et al. 2015; Dean et al. 1990; Eisner et al. 1977). This mode of defense is considered apomorphic in these two clades with respect to the general strategy of oozing or light spraying found in most carabids (Di Giglio et al. 2011).



**Fig. 1** A diagram showing a pygidial gland reservoir and reaction chamber of a bombardier beetle, adapted from (James et al. 2012). The pygidial gland reservoir contains a mixture of hydrogen peroxide and hydroquinones, which upon reservoir contraction are ejected into a sclerotized reaction chamber through a one-way valve. In the reaction chamber are enzymes produced by accessory glands which catalyze the degradation of hydrogen peroxide and convert hydroquinones to benzoquinones. The enthalpy of this process is great enough to cause a pressure gradient which expels the benzoquinones out of the abdomen at temperatures reaching up to 100 °C

**Defensive Chemical Diversity** Carabidae defensive compounds span 19 chemical classes and over 250 individual compounds, ranging from the simplest carboxylic acids to comparatively complex benzoquinones and monoterpenes (Table 1) (Lečić et al. 2014). Carabids have been shown to produce saturated and unsaturated carboxylic acids, most notably formic, acetic, methacrylic, and tiglic acids (Attygalle et al. 1991a; Blum et al. 1981; Dettner 1987; Eisner et al. 1977; Francke and Dettner 2005; Lečić et al. 2014; Moore and Wallbank 1968; Schildknecht et al. 1968a, b; Will et al. 2000). The presence of these acids appears to be sexually dimorphic in some species, with female beetles producing the unsaturated compounds while males produce the fully saturated form (Attygalle et al. 1991a). This is the only case of sexual dimorphism in Carabidae pygidial gland constituents that we are aware. It is possible, albeit untested, that sexual dimorphism in pygidial compounds could play a role in mediating behavior. Formic acid is generally accompanied by the co-occurrence of unbranched hydrocarbons in the pygidial glands as well, as is the case for Formicine ants where they are potentially used as a surfactant to enhance the effect of their formic acid defensive sprays (Will et al. 2000). Some subfamilies produce salicylaldehyde and other phenolic derivatives, though this is seemingly less common than the production of organic acids (Holliday et al. 2012; McCullough 1966). There have also been some reports of esters, aldehydes, ketones, terpenes, hydrogen cyanide, and others in certain taxa (Table 1) (Attygalle et al. 2009; Blum et al. 1981; Kanehisa and Murase 1977; Kanehisa and Kawazu 1985; Moore and Wallbank 1968; Schildknecht et al. 1964, 1968a, b). One chemical class that is of particular interest are the hydroquinones and their oxidized benzoquinone counterparts (Table 1). While quinones are present in four Carabidae subfamilies, Brachininae and Paussinae have evolved a unique way of utilizing them via rapid oxidation of hydroquinones to benzoquinones in a sclerotized reaction chamber (Fig. 1) (Balestrazzi et al. 1985; Schildknecht 1970; Schildknecht et al. 1968a, b).

In all cases so far mentioned, these chemicals represent those found in the adult development stage. Only two studies have examined glandular secretions of larval and pupal stages. When examining the larvae of *Chlaenius cordicollis*, Holliday et al. 2015 discovered that a gland which extruded in response to physical duress produced nine compounds, only one of which was found in the adult stage (2-methoxy-4-methylphenol). Six of these compounds, all quinones and phenolics, were suggested to be related to their adult counterparts by being their more oxidized versions, suggesting common biosynthetic pathways minus terminal reduction steps. 2-methoxy-4-ethylphenol was not suggested to be a precursor of any adult compounds by simple redox steps (Holliday et al. 2015). Di Giglio et al. 2009 discovered an enormous diversity of compounds in a single carabid species while examining the exocrine glands of

**Table 1** A table demonstrating common pygidial gland chemical classes produced by six major Carabidae subfamilies. Chemical structures are also presented for each compound in order of their listing

REPRESENTATIVE PYGIDIAL GLAND ALLOMONES OF SELECT CARABIDAE SUBFAMILIES			
Subfamily	Chemicals produced	References	Chemical structures
Brachininae	Quinones, hydrogen peroxide, hydrocarbons	Schildknecht et al. 1964, Moore & Wallbank 1968, Eisner et al. 1977, Kanehisa & Murase 1977, Eisner et al. 2001	
Carabinae	Salicylaldehyde, aliphatic organic acids	Eisner et al. 1963, Schildknecht et al. 1964, McCullough 1966, Moore & Wallbank 1968, Schildknecht 1970	
Cicindelinae	Benzaldehyde, hydrogen cyanide, aromatic acids, aliphatic esters, hydrocarbons	Blum et al. 1981, Pearson et al. 1988, Kelley & Schilling 1998, Schultz & Puchalski 2001	
Harpalinae	Aliphatic organic acids, quinones, phenolics, hydrocarbons, aliphatic ketones, aliphatic esters	Moore & Wallbank 1968, Kanehisa & Murase 1977, Kanehisa & Kawazu 1985, Attygalle et al. 1992, Will et al. 2000, Holliday et al. 2012	
Paussinae	Quinones, hydrogen peroxide, hydrocarbons	Moore & Wallbank 1968, Eisner et al. 1977, Roach et al. 1979, Aneshansley et al. 1983, Eisner et al. 1989	
Scaratinae	Quinones, terpenes, aliphatic organic acids, aliphatic ketones	Moore & Wallbank 1968, Kanehisa & Murase 1977, Moore & Brown 1979, Attygalle et al. 2009	

**Additional Information:**

1. Compounds shown for each subfamily do not represent an exhaustive list of compounds produced. Those represented here are amongst the most abundant or are biochemically unique within Carabidae
2. “R”, “R1”, and “R2” represent any functional group of a given length. Given the diversity of carabid compounds, the pygidial gland constituents can be best represented in terms of chemical classes
3. An R-group intersecting a bond within a ring indicates that the R-group may bind to multiple different carbons (i.e. there is more than one compound with that general structure)

*Carabus lefebvrei* pupae. In their glandular secretions, p-benzoquinone, two organic acids, numerous terpenes, aldehydes, and other compounds were detected; thirty-one compounds in total, two of which were isomers of  $\beta$ -ocimene (Di Giglio et al. 2009). Although these are only two studies, they exemplify

how diverse carabid defensive metabolomes can be not only across taxa, but within taxa across developmental stages. It is possible that some of these compounds play roles in mediating behavior between members of the same species, especially within aggregations (Fig. 2).



**Fig. 2** Hypothetical depiction of chemically-mediated tritrophic interactions between carabid beetles, a wolf spider, and a parasitoid. (a) Carabid spraying the wolf spider, a predator, with its pygidial gland allomones; (b) Pygidial gland allomones acting as alarm pheromones

for conspecifics; (c) A carabid beetle releasing an aggregation pheromone, which is detected by a conspecific at the other side of the rock; (d) A parasitoid wasp eavesdropping on volatile cues (kairomones) released by aggregating carabid beetles

**Non-exocrine Semiochemistry** Very few studies have examined carabid semiochemistry outside of the context of exocrine gland secretions. There are currently two accessible studies which examine cuticular chemistry, and there is only one that examines volatile emission from the entire beetle without exocrine gland emphasis. Bonacci et al. 2008 examined the cuticular chemistry of three carabid species: aposematic *Brachinus sclopeta* and *Anchomenus dorsalis*, as well as non-aposematic *Poecilus cupreus*, all of which can be found together in aggregations. It had been observed that *A. dorsalis* would exhibit a rubbing behavior toward *B. sclopeta*, which was initially suggested to be chemically-mediated and served as an odor-sharing mechanism (Brandmayr et al. 2006). *A. dorsalis* individuals were even observed to rub strips of paper that had been presented in monospecific *B. sclopeta* colonies, but not the control strips themselves. However, isolated individuals of *A. dorsalis* and *B. sclopeta* had very similar cuticular compound profiles compared to the non-aposematic co-aggregator, *Poecilus cupreus*. This suggested that most of the similarity seen was innate and due to the evolution of a Müllerian mimicry complex rather than odor sharing within an aggregation (Bonacci et al. 2008). However, it could be that the similarity in cuticular hydrocarbon profiles was acquired prior to individuals being separated for species-specific testing.

To investigate if cuticle chemistry is diet-dependent, Talarico et al. 2009 examined SPME extracts from a myrmecophilous beetle species, *Siagona europaea*, as well as one of their primary food sources, *Tapinoma nigerrimum*. It was discovered that beetles that had been fed ants resembled the cuticular chemical make-up of the ants more than unfed and isolated beetles. Fed beetles had acquired through some means an additional ten compounds compared to the fourteen found in unfed beetles,

all of which were also present in the ant prey. Most of these compounds were aliphatic ketones, alkanes, and unsaturated hydrocarbons, as well as iridomyrmecin and isopulegol, although many remained unidentified. The same general trend was observed in beetles that preyed upon *Messor capitatus*; when fed this ant species, the beetles acquired a total of sixteen compounds, doubling the eight that they had when isolated from ants. All identified compounds were alkanes, unsaturated hydrocarbons, as well as one ester. How these compounds are transferred from ant to beetle is currently unknown, although it is likely that they are acquired through physical contact rather than sequestration. (Talarico et al. 2009).

In contrast to the aforementioned non-volatile compounds characterized, Bonacci et al. 2011 was the first to examine the dynamic headspace volatiles released by carabid beetles, both placid and disturbed. In their analyses, they discovered that while each group produced only undecane, heneicosane, *Z*-9-tricosene, and tricosene, the disturbed beetles produced more of each compound, although only undecane was produced at a quantity that was found to be statistically significant (Bonacci et al. 2011).

## Potential Implications of Carabidae Pheromone & Kairomone Studies

**Allomones as Alarm Pheromones** Carabid beetles produce an extraordinary diversity of exocrine compounds, many of which have been described as allomones (Moore and Wallbank 1968; Schildknecht et al. 1968a, b; Will et al. 2000). While the efficiency of these sprays on predator deterrence has been assessed, the behavioral effects on the emitter and conspecifics have yet to be explored (Eisner et al. 2006; Eisner and Dean 1976), (Fig. 2). It

has been demonstrated in other insect orders that volatiles released from sprays, stings, and other defensive behaviors can elicit alarm behavior in conspecifics, and this may hold true for carabids. The dual use of allomones as alarm pheromones is considered *parsimonious semiochemistry* (Blum 1996). That is, it is suggested to be more parsimonious to use an honest signal of danger, an allomone, as an alarm pheromone than it is to have two separate compounds or blends of compounds for defense and alarm respectively. While this phenomenon of semiochemical bifunctionality is most often discussed in the context of eusocial insects, it has been also described in a number of solitary and gregarious species as well (Blum 1969, 1996; Cheng et al. 2017; Farine et al. 2002; Gunawardena and Bandumathie 1993; Lockwood and Story 1987; Löfqvist 1976; Machado et al. 2002; Wilson and Regnier 1971). Conspecific receivers of an alarm pheromone signal display one of two behavioral responses: (Alatalo and Mappes 1996) an increase in aggressive behavior toward the source of the threat, or (Albre et al. 2012) dispersal away from the source of the threat. These have been classically referred to as aggressive alarms and panic alarms, respectively (Wilson and Regnier 1971). In eusocial insects, the behavioral response may be caste specific, such as with the termite *Nasutitermes exitiosus*. Defensive sprays of *N. exitiosus* soldiers have been shown to recruit other soldiers to the emitter, while workers avoid the emission source if possible (Costa-Leonardo and Haifig 2010; Eisner et al. 1976). In the social wasp *Vespula velutina*, three aliphatic ketones released from the venom gland elicit an aggressive, stinging response in conspecifics (Cheng et al. 2017). In non-eusocial insects, such as the cockroach *Therea petiveriana* and the stink bug *Nezara viridula*, the seemingly universal response to these bifunctional defensive-alarm pheromones is dispersal (Farine et al. 2002; Lockwood and Story 1987). There is no documented behavior that we could find in the literature suggesting that non-social insects will risk predation to deter a threat when they themselves or their kin are not actively being attacked. That is, non-social insects are unlikely to risk predation to defend an unrelated conspecific. Thus, eusocial insects tend to respond to defensive-alarm pheromones as “unselfish swarms”, their specific behavior being taxa, caste, and context dependent, while non-social insects behave as selfish herds (Hamilton 1971; Young et al. 1994). This is to be expected, given that these eusocial insects have the genetic incentive as haplodiploids to protect their reproductive caste, even at the risk of being preyed upon or having their siblings preyed upon. Solitary insects generally have no such incentive, and thus disperse to retain the opportunity for reproduction or protect their kin (Hamilton 1964a, b, 1972; Trivers and Hare 1976).

Carabids offer an interesting opportunity to explore the role of defensive chemicals as alarm pheromones in a variety of ecological contexts (Fig. 2). While no carabid beetles are eusocial insects, certain taxa have been found to aggregate with conspecifics and, in some cases, heterospecifics under substrate (Bonacci et al. 2008; Philip and Burgess 2012; Thomas et al. 2001). Thus, carabids offer the unique opportunity to observe not only the response to defensive chemicals in solitary or gregarious species, but also between two to three different, co-aggregating species (Bonacci et al. 2008). This is especially true for the flanged bombardier beetles of the subfamily Paussinae, which are obligate or facultative myrmecophiles (Geiselhardt et al. 2007; Moore et al. 2011; Robertson and Moore 2016). It is currently unknown what behavioral effects their defensive blast of benzoquinones may have on their ant hosts, or what response formic acid or sting volatiles may elicit in the beetles. The behavioral effects that the defensive secretions of larvae and pupae may have on conspecifics is similarly a mystery.

**Kairomone-Mediated Parasitism** Kairomones also play a significant role in mediating behaviors between insects, particularly between hosts, parasites, and parasitoids (Brown et al. 1970). Although parasitism on carabids by other organisms is not a topic that has been investigated in great detail, there are reports of wasp, mite, and nematode parasitism occurring in this group (Andersen and Skorping 1990; Fain et al. 2009; Sasakawa et al. 2011). Most interactions between parasites/parasitoids and their hosts are chemically mediated to some extent, the parasitic taxa orienting to chemical cues released by the host either directly or indirectly. For example, *Cotesia marginiventris* (Braconidae) utilize herbivore-induced plant volatiles to find the general location of their *Spodoptera frugiperda* hosts. Once on the plant, the wasp will then use chemical signatures left by the caterpillars on the leaf to locate their host and parasitize them (Wölfling and Rostás 2009). Other parasitoids and parasites, such as the wasp *Aphytis melinus* and the nematode *Caenorhabditis japonica*, are attracted solely or primarily by volatile organic compounds released by the host itself rather than host-associated environmental signals (Okumura and Yoshiga 2014; Stelicht 1973). We know nothing, however, about the extent to which chemical eavesdropping on adult and immature carabid beetles by parasitoids and parasites may influence parasitic behavior (Fig. 2). One can only speculate on the diversity of parasites and parasitoids that afflict this hyper-diverse lineage, especially given that most preferentially infect one to a few species and may specialize on specific life stages (Althoff 2003; Brodeur 2000; Strand and Obrycki 1996).

Some carabid taxa are also reported as being ectoparasitoids on other organisms. Studies on North American *Brachinus* have shown that the larvae of some taxa are parasitoids of aquatic beetle pupae, notably within the

families Hydrophilidae, Gyrinidae, and Dytiscidae (Erwin 1967; Juliano 1984; Saska and Honek 2004). Since these pupae are nested within moist soil or under rocks obscuring them from view, there may be a chemical cue being released by the aquatic beetle pupae that is allowing the first-instar larvae or adult females to locate a host. Chemical eavesdropping on subterranean insects by surface-dwelling parasitoids has, to our knowledge, not been investigated before in insects. An investigation into the mechanisms regulating host-finding by *Brachinus* larvae could provide new insights into how parasitoids locate visibly hidden hosts against the noisy chemical background of the soil and the inhabitants therein.

*Brachinus* are not the only bombardier beetles to parasitize another species. Members of the subfamily Paussinae have obligate associations with ants and will prey upon them from within the colony (Moore et al. 2011; Robertson and Moore 2016). Their larvae employ this same predation tactic, neither seeming to evoke any usual aggressive response from their hosts (Geiselhardt et al. 2007). This is unusual behavior for ants to exhibit, a lack of aggressive response to a non-nest mate—even within a species, non-nest mates with the “incorrect” cuticular chemical profile are expelled or killed by worker or soldier castes (di Mauro et al. 2015; Emery and Tsutsui 2013; Martin et al. 2008; Sturgis and Gordon 2012). The ability of Paussine parasites to enter the colony and kill workers without evoking the colony’s aggression has been explained partially by their potential ability to mimic ant stridulations (Di Giulio et al. 2014). There is likely a chemical component to the mediation of this behavior, although no specific compounds have been isolated either from the beetle cuticle or glandular exudates from which ants feed (Geiselhardt et al. 2007). That in itself, the beetles providing a food source for the ants, could be enough to explain their protected status within the colony. Potentially, the beetles acquire cuticular compounds from constant contact with ants, or from consuming them, which would allow them to chemically blend in with their hosts (Elgar and Allan 2004; Johnson et al. 2001; Vander Meer and Wojcik 1982). They may also synthesize them independent of their hosts via convergence on a suite of biochemical pathways for producing cuticular constituents (Allan et al. 2002; Espelie and Hermann 1988; Geiselhardt et al. 2006). In some cases, myrmecophilous insects have been shown to release compounds that placate their ant hosts rather than attempting to blend in (Mori et al. 2000a, b; Visicchio et al. 1999). However, this is generally a short-lived behavioral modification that would not well-explain the beetles’ ability to remain in the nest for prolonged periods. It is also possible that they only possess a select few cuticular compounds that may be unimportant for nest mate recognition, essentially making themselves undetectable (Kleeberg et al. 2017). Other carabid taxa, such as larvae within the tribe Peleciini, are suggested to be ectoparasitoids of juvenile millipedes and Chrysomelid pupae (Erwin 1979; Salt 1928). The

genus *Lebistina* are also parasitoids of Chrysomelid pupae, and are interestingly used by members of the San tribe of Africa to poison-tip arrows (Weber et al. 2008). While it is true that most carabid larvae are free living scavengers, the ectoparasitic taxa mentioned here could serve as interesting focal species for research into chemically-mediated parasite-host interactions.

**Enhanced Understanding of Pheromone Diversity and Carabid Evolution** Animals communicate through a variety of mediums, including sight, sound, touch, taste, and smell (Bando 1991; Hallem et al. 2006; Jones and Teeling 2006; McDonald 1989; Sasahara et al. 2012; Weber 1973). In insects, pheromones are at the forefront of chemical communication between conspecifics (Hansson and Stensmyr 2011). Since the identification of the first insect sex pheromone, bombykol, by Adolf Butenandt in 1959, behaviorally-active pheromone constituents from thousands of insect species have been discovered, with more being discovered nearly daily (Butenandt et al. 1959; Symonds and Elgar 2007). However, despite the intensive work on Coleoptera pheromone characterization, there is no published literature regarding knowledge of pheromones in Carabidae (Francke and Dettner 2005). Pheromones have been shown to be incredibly diverse in their biosynthetic and evolutionary origins, chemical structure, blend complexity, and behavioral effects; thus, an analysis of pheromones in this group would provide key new insights into all of these aspects of biochemical evolution (Francke and Dettner 2005; Tillman et al. 1999; Yew and Chung 2015).

While many classes of pheromones have been behaviorally characterized in the literature, many chemical ecologists and ethologists have focused on sex pheromones, in certain contexts referred to as sex-aggregation pheromones (Cardé 2014; Landolt 1997). These compounds, as the name implies, positively mediate aggregative reproductive behavior between two individuals of the same species. Differentially produced by members of one, sometimes both biological sexes of a species, sex pheromones attract conspecifics of the opposite sex of the emitter, who may in turn be releasing a different pheromone constituent or blend into the immediate headspace (Cardé and Baker 1984; Xu et al. 2017). If pheromone constituents are detected by other conspecifics, aggregations may form due to a signaling cascade advertising reproductive opportunity (Landolt 1997). Thus, although they may never be directly detected by the olfactory receptors of conspecifics of the same sex, they may indirectly play a role in aggregation formation via the attraction of pheromone producing members of the sex opposite the emitter, and so on. These can be distinguished from aggregation pheromones *sensu stricto* which mediate aggregative behavior without necessarily playing a direct role in reproductive behavior (Cardé 2014). Rather, many aggregation pheromones are suggested to play primary

roles in communal feeding, such as with *Ips* and *Drosophila*, mate-finding being an indirect side-effect (Mast et al. 2014; Raffa 2011; Wertheim et al. 2005). Of course, microhabitats have a carrying capacity dictated by the resources available. Higher population densities also put those aggregating at a higher risk of acquiring disease or being parasitized if infected individuals are present, or being preyed upon (Dempster and Pollard 1981; Fuller et al. 2012; Ioannou et al. 2009). To ensure that the signal cascade produced by the positive feedback loop of pheromone release and attraction doesn't lead to overcrowding, some insects will release anti-aggregation pheromones. As their name implies, these function similarly to allomones, deterring in this case conspecifics (hence their classification of "pheromones") to maintain appropriate population densities. This particular pheromone-induced anti-aggregation effect has been particularly well-studied the bark beetles (Bakke 1981; Borden et al. 1987; Miller et al. 1995, 2005). Given that gregarious behavior is present in many carabid taxa, whether it be for increased fecundity, feeding, defense, etc., it is conceivable that pheromones may be playing a significant role in the formation and maintenance of such aggregations (Alatalo and Mappes 1996; Gamberale and Tullberg 1998). While they would etymologically not be classified as pheromones given the context, there may be a chemical basis to the mediation of interspecific aggregation formation and maintenance as well (Walgenbach et al. 1982). Given the potential importance of these aggregations during periods of inactivity and their relevance to the natural history of this group, we suggest that further investigation into these gregarious behaviors are warranted (Arndt et al. 2005).

When sex pheromones, whether they be blends or single constituents, are released at the proper concentrations, ratios, and time, they may serve as the lynch-pin for ensuring reproductive success. If there is even the slightest phenotypic variation in any of these factors, especially in the chemical makeup of a blend, the signal and expected behavior become desynchronized (Baker 1998; Kárpáti et al. 2013; Klun et al. 1973). That is, an inappropriate signal will not likely elicit the preferred response, such as attraction for mating. If this desynchronization is prevalent in a great enough proportion of the population in question, and if this truly does cause ethological reproductive isolation, speciation events may begin to occur (Wicker-Thomas 2009; Nanda and Singh 2012). These alterations to wild-type pheromone blends are generally the result of altered enzymatic activity, whether it be in terms of the quantity of pheromone constituents produced, their relative ratios, or chemical identity (Albre et al. 2012; Buček et al. 2015). In many Lepidoptera, primarily moths, most sex pheromones are synthesized from fatty acid precursors through a series of  $\beta$ -oxidation and desaturation reactions (Roelofs and Bjostad 1984). They may then be modified via acetylation, carbonyl reduction, or other biochemical steps to form the final pheromone product (Bjostad and Roelofs 1983;

Roelofs and Rooney 2003). In the European corn borer, *Ostrinia nubilalis*, two distinct chemotypes are present throughout the Midwestern and Northeastern United States. The Midwestern population primarily produce a 97:3 blend of Z:E-11-tetradecenyl acetate (TDA), while the Northeastern population produce a 3:97 blend of Z:E-11-TDA. Behavioral assays show that male moths of one chemotype are often not significantly attracted to pheromone blends of the other (i.e. New York *Ostrinia* are seldom attracted to the pheromone blends of Iowan moths, and *vice versa*). However, in regions where the ranges of both chemotypes overlap (e.g. Pennsylvania), males were attracted to a 1:1 blend of the two pheromone isomers as well as the 97:3 and 3:97 blends (Klun et al. 1975; Kocbansky et al. 1975; Roelofs et al. 1985). It is conceivable that sustained ethological reproductive isolation over a long enough period of time may lead to the buildup of genetic differences such that speciation events occur (Jennings et al. 2011, 2014). Much of the early work on pheromone evolution was also conducted in the genus *Ostrinia*, particularly using *O. nubilalis* and *O. furnacalis*, the European and Asian corn borers respectively. Both species produce structurally similar primary pheromone constituents, Z/E-11-TDA and Z/E-12-TDA respectively; a desaturation shift on either side of the twelfth carbon of the alkyl chain. In both *Ostrinia* species,  $\Delta 9$ ,  $\Delta 11$ , and  $\Delta 14$  desaturases were detected in the abdominal pheromone production glands. However, only  $\Delta 11$ -produced pheromone product (Z/E-11-TDA) was found in *O. nubilalis* whereas *O. furnacalis* produces the  $\Delta 14$  pheromone product (Z/E-12-TDA). Thus, somewhere in the evolutionary history of *Ostrinia*, a switch to the utilization of  $\Delta 14$  occurred, leading to the observed chemotype of *O. furnacalis* (Roelofs et al. 2002). It was hypothesized that this may have occurred via the resurrection of a  $\Delta 14$  pseudogene combined with some suppression of  $\Delta 11$ -desaturase transcription or enzymatic activity in this lineage. Similar examples of pheromone evolution and the role that it can play in the process of organismal evolution have been identified in other insects (Baker 2002; Ferveur 2005; Symonds and Elgar 2003). If studies into the genetic and biosynthetic bases of pheromone evolution are undertaken in carabids, along with work into the neurophysiology and pheromone-mediated behavior of this group, we could gain valuable insights into how this group has radiated to the extent that it has should pheromone evolution be playing a significant role.

The Carabidae are one of the single largest families of insects currently known (Arndt et al. 2005). Members of this family have colonized nearly every habitat on land, and in rare cases, some have become semi-aquatic, such as the Fairy Shrimp Hunting Beetle, *Cicindis horni* (Erwin and Aschero 2004). One fossilized species has even been found in Antarctica from the early to mid-Miocene (14–20 Ma), and many extant species inhabit the arctic tundra (Ashworth and

Erwin 2016; Garry 1993). Such biodiversity has captivated the attention of biologists and numerous mechanisms have been suggested to play a role in speciation. One such mechanism is reproductive isolation, be it allopatric or sympatric (Bolnick and Fitzpatrick 2007; Jennings et al. 2011, 2014). Given that pheromone evolution has been shown to be saltational in some cases, sympatric reproductive isolation via a sudden desynchronization in pheromone emission, olfaction, and behavior is one way in by which speciation events can occur (Baker 2002; Ferveur 2005; Nanda and Singh 2012; Symonds and Elgar 2003; Wicker-Thomas 2009). A broad investigation into the pheromone constituents of this family, their biosynthesis pathways, and the rates of evolution of the enzymes involved is necessary to make any inferences as to what role saltational shifts in pheromone blends may have had in the diversification of this lineage of beetles.

It is possible that we have unknowingly detected pheromone components in the defensive secretions and sprays of the carabid species examined thus far (Lečić et al. 2014). As previously mentioned, many defensive allomones may also serve roles as alarm pheromones, being that they are an honest signal of danger (Blum 1969, 1996; Eisner et al. 1976; Löfqvist 1976). However, in some cases, allomones may serve a dual role as sex pheromones. In the parasitoid wasp, *Leptopilina heterotoma*, the defensive compound (–)-iridomyrmecin functions not only as a defensive allomone, but also as a competition avoidance cue in the context of reproduction (Weiss et al. 2013). In the presence of other minor constituents such as (+)-isoiridomyrmecin and other structurally related compounds, it has also been found to function as a sex pheromone constituent (Weiss et al. 2013). Although this type of semiochemical parsimony has not been widely reported on in the literature, it may be more prevalent than we are currently aware (Geiselhardt et al. 2008; Ruther et al. 2001; Weiss et al. 2013). Given the diversity of natural histories, as well as the diversity of their defensive allomones, it may be carabids also employ semiochemical parsimony (Fig. 2). A natural first target for studies into alarm pheromone emission, detection, and chemically-mediated alarm behavior within the Carabidae would be primary pygidial gland defensive secretions. Not only are these compounds easily extractable from the reservoirs, most are also commercially available or easily synthesizable, such as benzaldehyde produced by members of the Cicindelinae (tiger beetles). An added benefit to utilizing the Carabidae as a model for studying alarm pheromone behavior is that the gland constituents are relatively simple within a species, most taxa usually only containing one to a few major compounds and occasionally a small number of minor constituents. While the usual challenges of running successful behavioral assays on insects would persist, many steps related to the identification of multiple compounds produced in trace quantities would be eliminated due to the high quantity and relative homogeneity of intraspecific gland constituents in certain taxa.

## V: Next-Generation Chemical Ecology

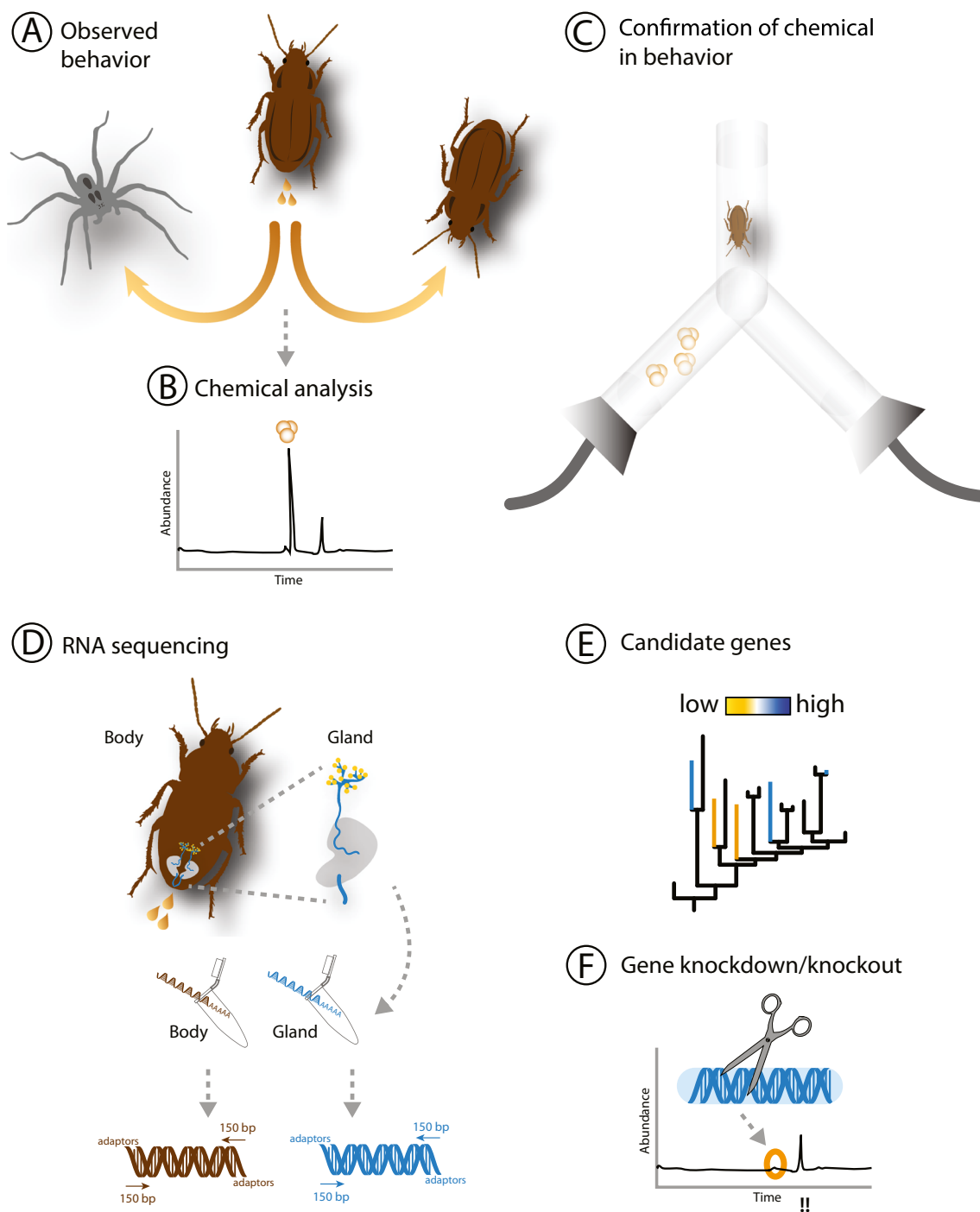
Over the past 60 years, our ability to identify insect semiochemicals has greatly improved. More than 3500 unique compounds have been discovered in thousands of taxa, belonging to nearly every insect order (El-Sayed 2018). Many of these compounds have been assigned semiochemical classes and are hypothesized to have specific influences on insect behavior based on the ecological conditions of their emission. In contrast, the underlying genetics of semiochemical biosynthesis in many insects remain understudied, which is reflected by a lack of genetic data for many insect groups including carabids. Now, with the advent of next-generation sequencing technologies, many of the financial and logistical barriers that previously impeded the high-throughput study of genes involved in insect semiochemical biosynthesis have been greatly diminished. Studies can be conducted to identify genes involved in physiological processes for whole insect bodies, specific glandular tissues, and even individual cells (Ziegenhain et al. 2017), allowing for high-resolution analyses of differential gene expression between or within tissues. Integrating next-generation sequencing with analytical chemistry and biochemical techniques can allow for an enhanced understanding of semiochemical production and evolution throughout Insecta. To advance understanding of carabid semiochemistry specifically, we suggest an integrative approach that combines tissue-specific transcriptomics with spectroscopic and biochemical assays to elucidate complete biosynthetic pathway(s) for semiochemicals.

Next-generation sequencing technologies have revolutionized the method by which gene expression can be studied within an organism, tissue or cell. In particular, whole transcriptome sequencing, also known as RNA sequencing (RNA-Seq), can be used to analyze context-dependent gene expression utilizing billions of sequenced base pairs (Conesa et al. 2016). For example, transcript abundance can be used to identify genes that are differentially expressed between samples (e.g. tissue of interest relative to the whole body) and have putative functions in tissue-specific biosynthetic processes. RNA-Seq has already proven to be a successful method for the identification of candidate genes involved in pheromone biosynthesis (Buček et al. 2015, 2016; Nadeau et al. 2017; Vogel et al. 2010). For instance, transcriptome sequencing of the *Heliothis virescens* pheromone gland revealed high expression of several different candidate genes, which may play roles in pheromone biosynthesis (Vogel et al. 2010). Many of these were annotated as alcohol and aldehyde oxidases, fatty acid synthetases and reductases, and various fatty acid desaturases, which corroborates findings from previous studies on the biosynthesis of noctuid pheromone constituents that these genes are involved in pheromone biosynthesis (Teal and Tumlinson 1986, 1988). Similarly, RNA-Seq may be a promising tool for the identification of genes expressed in carabid tissues that biosynthesize



semiochemicals, which could then serve as candidates in studies investigating biosynthesis (Fig. 3).

While RNA-Seq can provide support for tissue-specific patterns of gene expression, this method cannot confirm



**Fig. 3** Workflow showing how methods from ethology, chemistry, and molecular biology can be integrated toward the goal of elucidating the evolution of chemically-mediated insect-insect interactions. **(a)** Behavioral observations are made of a carabid beetle spraying pygidial gland contents, which are detected by a spider (allomone) and a conspecific (alarm pheromone). **(b)** Interested in what chemicals are mediating this interaction, GC-MS analyses are first conducted on gland extracts. **(c)** Behavioral assays are then conducted using individual gland constituents or blends to characterize their role in

behavior (if any). **(d)** RNA is extracted from glandular and non-glandular tissue and is sent for transcriptome sequencing. **(e)** Differentially expressed candidate genes that may be involved in the biosynthesis of behaviorally active compounds are identified. Phylogenetic trees of gene families may be reconstructed to infer the evolution of semiochemical production within a clade. **(f)** Gene knockdown or gene knockout is used to confirm the role of candidate gene products in the biosynthesis of the semiochemical of interest. Subsequent analyses could be conducted as dictated by research interests

which transcripts are functionally relevant for a given metabolic process (Fig. 3). Two promising techniques in studying the role of gene products in the biosynthesis of semiochemicals are RNA interference (RNAi) and CRISPR/Cas9, which can be used to knock down or knock out gene expression (Cong et al. 2013; Fire et al. 1998). The result is an observable phenotype, such as the absence or reduced production of a chemical constituent observable via gas chromatography–mass spectrometry (Li et al. 2013). Thus, from a set of differentially expressed genes, possible precursors, cofactors, and potentially even intermediates in the pathway can be inferred at least in-so-far as it narrows down the list of candidates involved. However, with CRISPR/Cas9, it is necessary for injections to be made in the egg stage of the insect, and thus a well-maintained colony is required (Kistler et al. 2015). Where this is unrealistic due to the complex life history strategies of many insects and difficulty of rearing, RNAi may serve as an alternative for assessing gene function, as it can be used at various life stages once optimized.

Radiolabeled precursor incorporation followed by candidate enzyme characterization should be combined with gene knockdown and knockout methods to assess whether genes expressed within carabid pygidial glands play a role in converting chemical precursors to semiochemical constituents. A prime example of such *in vivo* radiolabeled assays comes from Attygalle et al. 1991b, in which the injection of D<sub>8</sub>-L-valine into the carabid beetle *Scarites subterraneus* was shown to be highly incorporated into methacrylic acid and isobutyric acid, two pygidial gland allomones, via GC-MS analysis of gland extracts (Attygalle et al. 1991b). An alternative to injections is the incorporation of radiolabeled precursors into the diet, which is particularly useful if the insect is too small to handle injections or if the stress and physical damage induced by a needle is undesirable. This method was historically popular in studies examining the essential amino acid requirements of insects (Kasting and McGinnis 1958; Rock and King 1966). The same method of feeding an insect radiolabeled candidate precursors, preferably those more immediately upstream of the compound(s) of interest, could also reveal which if any are incorporated into, for example, a carabid semiochemical constituent.

To validate the role of candidate genes in carabid semiochemical biosynthesis, protein expression followed by enzymatic assays can be performed on individual steps in biosynthetic pathways. For example, expression vectors can be designed from the candidate genes discovered via RNA-Seq for their introduction into cell lines (Ohlen et al. 2016; Steiner et al. 2018; Zhang et al. 2018). Once the purified protein isolate has been acquired, physiological conditions can be replicated by adding appropriate concentrations of relevant cofactors and the substrate of interest (Ohlen et al. 2016; Steiner et al. 2018; Zhang et al. 2018). The reaction can

essentially be performed *in vitro*, and the conversion of substrate to product can be confirmed using GC-MS analysis. By conducting such assays, candidate genes identified in RNA-Seq experiments can be functionally validated via the confirmation of their role in semiochemical biosynthesis at the enzyme-substrate level.

To gain a deeper understanding of the processes by which semiochemical biosynthetic pathways evolve and diversify, the evolutionary history of the gene families involved should also be considered. A previous study of Lepidopteran fatty acid desaturases, which are important genes for pheromone biosynthesis in this order, serves as a prime example of how tissue-specific RNA-Seq can be combined with phylogenetics and molecular evolutionary analyses. Phylogenetic reconstruction of the Lepidopteran fatty acid desaturase gene family revealed interesting implications for pheromone evolution in moths, including the existence of many functionally conserved, yet inactive clades that lie dormant within moth genomes (Buček et al. 2015). In addition, single point mutations were suggested to alter the substrate specificity of two paralogous enzymes, a Z11-desaturase/conjugase and an E/Z-14 desaturase in *Manduca sexta* (Buček et al. 2015). This shift in specificity via the mutation of a small number of amino acids was suggested to be a potential mechanism by which novel pheromone constituents could evolve. Similar studies in Carabidae have the potential to reveal interesting gene birth, death, and functional divergence events associated with shifts in semiochemical production (Fig. 3).

## Conclusions

The exocrine chemistry of Carabidae have been investigated heavily over the past 60 years, and thanks to the efforts of many dedicated carabidologists and chemical ecologists, we have amassed a rich body of allomone literature across the family (Moore and Wallbank 1968; Will et al. 2000; Schildknecht et al. 1968a, b). As important as this is to understanding the evolution of chemical defensive strategies, it could be complimented by studies of other chemically-mediated methods of intraspecific and interspecific communication. Without knowledge of how carabids mediate their complex interspecific aggregations, find hosts for predation deep within volatile-rich soil, survive the selective chemical screening by ant colonies, and ultimately find their mates in a complex world of chemical and visual cues, we leave much unknown about this ecologically important and diverse lineage. In addition, understanding the biosynthesis of carabid semiochemicals and the evolution of the complex behaviors that they mediate is arguably as necessary as understanding the chemicals and behaviors themselves. Few studies have elucidated the underlying genetics of semiochemical biosynthetic pathways in insects, especially within Carabidae.

Therefore, we strongly support expanding studies of carabid semiochemistry, olfaction, and behavior.

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