ABSTRACT

HAMILTON, JAMORA ANICE. An Investigation of German Cockroach Aggregation Behavior and the Implications for Pest Management. (Under the direction of Dr. Coby Schal).

The German cockroach, *Blattella germanica* L. (Blattodea: Ectobiidae), is a worldwide pest widely associated with human structures. Infestations are often found in homes where they can contaminate food and surfaces with pathogens and produce allergens that cause asthma, allergic reactions, and other health problems. German cockroaches also form aggregations that intensify these conditions. Over the years, management of this pest has relied mainly on broad spectrum insecticides and more recently the use of insecticide baits. However, with the evolution of physiological resistance to various insecticides, it is necessary to continue to research the behavior of the cockroach and improve current management techniques. In the following studies, I investigated compounds that drive aggregation behavior and examined how aggregation and related cockroach behaviors can be exploited to enhance pest management strategies.

The source and identity of the German cockroach aggregation pheromone has been heavily debated. We investigated the role of cuticular hydrocarbons (CHCs) in the feces and on the cockroach body as aggregation signals. We extracted CHCs from adult females and purified them using flash chromatography. CHCs and other compounds from feces-conditioned filter papers were extracted using various solvents. Two-choice behavior assays were performed with first instar nymphs and CHCs were either applied to filter paper shelters, representing fecal deposition, or to previously extracted cockroaches, representing shelter co-habitation with other cockroaches. We found that nymphs preferred to rest in shelters that contained a CHC-free dead female, and the addition of CHCs to the female did not enhance aggregation. In these

assays, purified CHCs did not elicit more aggregation and played a minor role in aggregation behavior. A methanol extract of feces was highly effective, which was consistent with results from studies showing that volatile carboxylic acids produced by gut microbes in the feces act as aggregation agents. We manipulated the gut microbiomes of cockroach colonies with different diets and fecal inocula and assessed their aggregation preferences in choice bioassays. Our results showed that nymphs preferred aqueous extracts of their own colony feces over fecal extracts from colonies that fed on a different diet. These findings suggest that unique gut microbial communities in each colony produce colony-specific aggregation signals that guide colony recognition.

The spread of microbes within a colony occurs mainly through coprophagy, where cockroaches feed on the feces of conspecifics. This behavior has been shown to facilitate the systemic horizontal transfer of insecticides, a phenomenon known as secondary kill. Our goal was to determine whether horizontal transfer can go beyond secondary, to tertiary and quaternary effects and to compare various gel baits with different active ingredients. We found that all the tested baits caused 100% mortality of adult females that directly fed on the bait and relatively high secondary mortality of first instar nymphs. Baits containing dinotefuran, emamectin benzoate, fipronil or indoxacarb caused tertiary mortality, but only the fipronil and indoxacarb baits caused low quaternary mortality. By designing baits that exploit these behaviors, we can potentially develop gel baits that can attain higher levels of mortality to suppress cockroach populations.

Because German cockroaches have developed resistance to active ingredients in some gel bait products, there is a need to incorporate insecticides with diverse modes of action. We evaluated the potential utility of novaluron, a chitin synthesis inhibitor, in bait formulations against the cockroach. We found that novaluron was effective both by topical application and by ingestion. At concentrations of 100 ppm and higher, novaluron caused mortality in nymphs and interfered with ootheca production in adult females, but only before they formed an ootheca. It successfully reduced German cockroach populations in cages and has potential to be incorporated in bait rotation plans to manage insecticide resistance.

An Investigation of German Cockroach Aggregation Behavior and the Implications for Pest Management

by
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DEDICATION

To my mother, Gale Hamilton, and to my father, Gregory Hamilton, for your constant encouragement, support, and love. To my sister, Dr. Jameaka Hamilton, for being an exceptional role model. To my husband, Willie Brown, thank you for always believing in me.

BIOGRAPHY

Jamora Anice Hamilton was born in Hampton, VA and grew up in Columbia, SC. She attended Clemson University where she earned her B.A. in Chemistry. Her growing interest in insect behavior led her to pursue her Ph.D. in Entomology at North Carolina State University.

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CHAPTER 1

Introduction

German Cockroach Biology, Behavior, and Ecology

Adult German cockroaches, *Blattella germanica* L. (Blattodea: Ectobiidae), range in color from tan to dark brown and have two parallel black stripes on their pronotum running from the back of the head to the base of the wings. They are typically 10-15 mm long, with males having a slender body and tapering abdomen and females having a more robust body and rounded abdomen (Rust et al. 1995). Unlike many other cockroach species, female German cockroaches retain their oothecae and either carry the egg case externally until eggs hatch or drop it within a short time before hatch (Barson and Renn 1983). The immature nymphs are mostly black with some light coloration, and have distinct parallel stripes like the adults. There are five to six instar stages and nymphs develop larger wing pads as they mature after each molt.

Public Health Significance

German cockroaches are an economically and medically important synanthropic pest with worldwide distribution (Schal and Hamilton 1990, Rust et al. 1995), and approximately 26% of the U.S. population has developed some sensitivity to these cockroaches (Arbes Jr et al. 2005). They are almost exclusively indoor pests that are associated with human-built structures, especially in food preparation areas like restaurants, food processing facilities, schools, and homes where they can be serious threats to human health.

Cockroaches are potential vectors of pathogenic organisms and have been shown to carry diverse pathogenic and non-pathogenic bacterial flora, different protozoa, fungi, and viruses (Fotedar et al. 1989, Fotedar et al. 1991, Kopanic Jr et al. 1994, Tatfeng et al. 2005, Bennett 2008). They can readily move from contaminated areas and create the opportunity to spread disease-causing organisms on food and surfaces, and it was reported that 98% of cockroaches found in medical facilities could carry pathogens on their integuments or in their digestive tracts (Cloarec et al. 1992). German cockroaches in particular pose a serious risk to human health by significantly contaminating the environment. It was also demonstrated that *Salmonella* spp. were able to survive and multiply in their gut and feces (Ash and Greenberg 1980), and that this pathogen could be passed to other cockroaches and objects (Kopanic Jr et al. 1994). While many studies have highlighted a possible risk of these cockroaches as vectors of human or animal diseases, their role in the direct transmission of infection has seldom been established and is somewhat uncertain (Menasria et al. 2014).

Cockroaches are also major sources of indoor allergens; however, only allergens from the German cockroach and American cockroach (*Periplaneta americana* L.) have been officially recognized and named according to the World Health Organization/International Union of Immunological Societies nomenclature (IUIS 2017). Large amounts of *Blattella germanica* allergen 1 (Bla g 1), a major allergen produced by the German cockroach, is excreted in the feces and a single adult female has the capacity to produce a level of Bla g 1 that far exceeds the proposed human sensitization and morbidity thresholds for exposure (Gore and Schal 2004). Sensitization and exposure to these allergens has been associated with the development and exacerbation of acute asthma morbidity and increased medical utilizations, especially among inner city children (Kang et al. 1979, Call et al. 1992, Gelber et al. 1993, Rosenstreich et al. 1997, Gore and Schal 2007). Allergen removal is accomplished through vacuuming to remove contaminated dust, dead cockroaches, and their feces from structures and cleaning to remove allergen from floors, bedding, and other surfaces (Gore and Schal 2007). In addition to sanitation, pest control is a pivotal component in cockroach allergen mitigation programs, and it has been shown that management of cockroach populations can significantly reduce the level of cockroach allergen in infested homes (Gore and Schal 2007, Sever et al. 2007).

Aggregation Behavior

German cockroaches are generally most active at night and return to the harborage during the day, forming large groups or aggregations (Schal et al. 1984, Rust et al. 1995, Bell et al. 2007). Aggregations are beneficial to the cockroach by accelerating nymphal development (Willis et al. 1958) and reproductive maturation in adults (Uzsák and Schal 2012, 2013), and by facilitating mate location, predator avoidance, thermoregulation (Appel et al. 1983, Parrish et al. 1997), and prevention of water loss (Dambach and Goehlen 1999). Aggregation behavior is driven by pheromones, which are semiochemicals secreted by individuals that are perceived and analyzed by conspecifics to orient their behavior (Wyatt 2003). Previous studies have suggested several chemicals as aggregation pheromones, including rectal pad secretions (Ishii and Kuwahara 1967), short- and medium-chain volatile fatty acids in feces (Ritter and Persoons 1974, McFarlane and Alli 1986, Wada-Katsumata et al. 2015), alkylamines and blattellastanoside A and B (Sakuma and Fukami 1990, 1993), and cuticular hydrocarbons (Rivault et al. 1998). Salivary components cause cockroaches to disperse from aggregations (Ross and Tignor 1985).

German cockroaches are omnivorous scavengers that are well adapted to feeding on a variety of food materials (Rust et al. 1995). First instar nymphs are less likely than older nymphs and adults to forage long distances from a shelter for food and are more likely to participate in coprophagy within aggregations (Cloarec and Rivault 1991). Coprophagy involves feeding on the feces of other conspecifics and provides essential nutrients and microbiota to nymphs (Kopanic Jr and Schal 1999, Nalepa et al. 2001). Coprophagous behavior in cockroaches most likely originated because feces are concentrated sources of microbial communities functionally similar to those living on ingested plant matter, and as mutualistic relationships between the insect and microbes developed, coprophagy of conspecific fecal material would become a mechanism for ensuring that neonates received an inoculum of the appropriate gut fauna efficiently (Nalepa et al. 2001). The omnivorous and coprophagous habits of cockroaches are likely to generate highly variable gut microbiomes that emit different blends of fecal volatile carboxylic acids, leading to a preference for compounds produced by the local gut bacterial community and thus may drive aggregation preference and fidelity to specific aggregation sites (Wada-Katsumata et al. 2015).

Baits in Cockroach Management and their Horizontal Transfer in Aggregations

The application of insecticide sprays to control German cockroaches was the principal method used until the early 1990s when the use of slow-acting baits became increasingly more important in the United States (Gooch 1999, Hedges 1999). Current integrated pest management programs for cockroaches involve using inspections and monitoring to identify and prevent conditions that are conducive to cockroach infestations (available food and water sources or harborages). It is important to detect infestations early, and pinpoint cockroach aggregations. Insecticides are only used as needed, with reduced-risk formulations such as baits, being preferred. Gel baits can be applied in tight areas like cracks and crevices to target the aggregations and shelters that have been identified during inspections, thus increasing the bioavailability of the insecticide treatments to cockroaches while minimizing exposure of building occupants (Nalyanya et al. 2014).

Gel bait formulations of insecticides have been shown to be convenient to use and highly effective in managing German cockroach populations. Baits offer greater efficacy and specificity, are considered safer and more environmentally friendly due to their targeted application, can have a variety of active ingredients with diverse modes of action incorporated into their formulations, and can be used in insecticide-sensitive areas compared to insecticidal sprays (Buczkowski et al. 2001, Appel 2003, Gondhalekar et al. 2011, Schal et al. 2011). Baits have also been found to provide systemic translocation of insecticides to individuals in a population, resulting in mortality of cockroaches that do not visit the bait, termed secondary kill or secondary mortality (Silverman et al. 1991, Kopanic and Schal 1997, Durier and Rivault 2000, Buczkowski and Schal 2001, Buczkowski et al. 2008). Secondary mortality was first reported in in the German cockroach using radiolabeled hydramethylnon (Silverman et al. 1991). Several cockroach behaviors have been shown to facilitate the horizontal transfer of insecticides, including contact (Durier and Rivault 2000, Buczkowski and Schal 2001), coprophagy (Silverman et al. 1991, Kopanic and Schal 1997, Kopanic Jr and Schal 1999, Buczkowski et al. 2001), emetophagy (Buczkowski and Schal 2001), and necrophagy and cannibalism (Gahlhoff Jr et al. 1999, le Patourel 2000, Buczkowski et al. 2008).

Through many years of pesticide use, the German cockroach has developed resistance to nearly every class of insecticide (Tang et al. 2019). Behavioral resistance through aversion to the bait ingredient glucose was reported (Ross 1993, Silverman and Bieman 1993, Silverman and Ross 1994, Ross 1998), and accounted for the failure of a cockroach bait product in the 1990s (Silverman and Bieman 1993). There have also been reports of avoidance to gel baits containing the phagostimulants fructose, glucose, maltose, and sucrose (Morrison et al. 2004, Wang et al. 2004, Liang 2005, Miller and McCoy 2005, Wang and Bennett 2006a). When active ingredients are incorporated into a palatable bait, cockroaches readily consume a lethal dose from a single meal, reducing the likelihood of selection for high-level cockroach resistance (Wang et al. 2004). These findings suggest that rotation of both bait and active ingredients is necessary to delay resistance in German cockroaches.

Insect Growth Regulators in Cockroach Management

Insect growth regulators (IGRs) have been used in pest management since the 1980s. IGRs are compounds that interfere with physiological and biochemical processes for normal growth, development, and reproduction in insects, either by creating an imbalance in certain hormones and enzymes or interfering with their actions, causing abnormalities that impair survival or reproduction at a particular point in the insect's life cycle (Retnakaran et al. 1985, Rust et al. 1995, Pener and Dhadialla 2012). These compounds are compatible with integrated pest management programs because they are specific to the target pest species, have minimal effects on beneficial insects, and have unique modes of action that are different from conventional broad-spectrum (neurotoxic) insecticides (Hajjar 1985, Retnakaran et al. 1985, Pener and Dhadialla 2012). The IGRs that have received the most attention for management of German cockroaches are grouped according to their mode of action, either as chemicals that mimic key insect hormones (juvenile hormone analogs [JHAs], ecdysone agonists), or interfere with cuticle formation (chitin synthesis inhibitors [CSIs]).

JHAs cause delays in development, increased melanization or coloration, disrupted regeneration of appendages, molt inhibition (leading to mortality), developmental anomalies in reproductive organs and other structures which affect reproduction, and morphological anomalies in sensory organs which alter behavior (Staal 1975, Sehnal 1983). German cockroaches exposed to JHAs in the final nymphal stadium develop into "adultoids" that possess distinctive morphogenetic deformities such as twisted, curled, or crinkled wings (Riddiford et al. 1975, King and Bennett 1991). This exposure also causes deformities in male genitalia, preventing copulation and sperm transfer (Staal et al. 1985, Brenner et al. 1988, King and Bennett 1989), and physiological defects in the ovaries and colleterial glands of females, inhibiting reproduction (Das and Gupta 1974, Staal et al. 1985, Kramer et al. 1989). While they have no immediate effect on cockroach survival, use of JHAs in cockroach management is focused on controlling the population by disrupting normal reproduction and limiting population growth.

CSIs inhibit the synthesis of chitin, a major component of the insect cuticle, and interfere with deposition of the new cuticle, leading to and death at or after the next molt (Rust et al. 1995, Pener and Dhadialla 2012). The chitin synthesis inhibitors that are derivatives of benzoylphenyl ureas (BPUs) have been extensively studied as insect control agents since their discovery in the 1970s (Mulder and Gijswijt 1973, Hajjar 1985). BPUs are most active as stomach poisons (Mulder and Gijswijt 1973), but activity by contact has also been reported (Ross and Brady 1983). BPUs inhibit chitin biosynthesis, disrupt chitin deposition and cause affected insects to form weak integuments and die due to complications which arise from molting (Mulder and Gijswijt 1973, Hajjar and Casida 1978, Vincent 1978, Van Eck 1979). They also possess ovicidal activity by disrupting cuticle formation in developing embryos and causing a failure to hatch. However, ovicidal activity in cockroaches is limited to females exposed to BPUs while developing an ootheca (Weaver et al. 1984, Yonker and Bennett 1986, Koehler and Patterson 1989, DeMark and Bennett 1990). Several BPUs have been studied for their potential in cockroach management and have been shown to be effective in reducing populations. They were found to cause death of German cockroach nymphs during ecdysis (Ross and Brady 1983, Weaver et al. 1984, Shafi et al. 1987, Demark and Bennett 1989, Koehler and Patterson 1989, Reid et al. 1992, Mosson et al. 1995, Kaakeh et al. 1997, Ameen et al. 2005, Wang and Bennett 2006b), and disrupt cuticle formation of embryos, preventing hatch of neonates (Hajjar 1985, Retnakaran et al. 1985, Yonker and Bennett 1986, DeMark and Bennett 1990, King 2005).

IGRs that have been registered for German cockroach control are mostly JHAs, even though CSIs have been shown to be effective in managing cockroach populations. CSIs may suppress cockroach populations faster than JHAs (Koehler and Patterson 1989, Rust et al. 1995) because CSIs affect all stages that molt (including embryos) whereas JHAs mainly target the final nymphal molt. CSIs also can be used in combination with other active ingredients that serve as adulticides, so that all life stages of the German cockroach are targeted, and they can be incorporated into baits to manage insecticide resistance.

Focus of Dissertation

German cockroaches are the most prevalent and important indoor cockroach pest in the United States (Rust et al. 1995). While there are numerous tactics and technologies available to effect suppression of indoor cockroach populations, there is still reliance on the application of insecticides, especially as spray formulations. As concerns about indoor insecticide use rise among the public and pest control professionals, there is a continued need for research on the implementation of reduced-risk strategies for cockroach control. The overall goals of this dissertation are to investigate compounds that drive German cockroach aggregation behavior, examine how aggregation and related cockroach behaviors can be utilized to enhance current pest management strategies, and explore the use of alternative insecticides in cockroach management.

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CHAPTER 2

Role of Cuticular Hydrocarbons in German cockroach (Blattodea: Ectobiidae) Aggregation Behavior

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Abstract

Aggregation can be adaptive by providing protection from predators, facilitating thermoregulation, and expediting the location of food, shelter, and mates. German cockroaches (*Blattella germanica*) are obligatory commensals in human-built structures, where they aggregate in crevices during the day. The source of the aggregation pheromone that drives this behavior and its chemical identity remain unclear. Cuticular hydrocarbons (CHCs) in feces have been proposed to serve as aggregation pheromone, but this function has not been investigated in relation to visual and tactile cues that mediate aggregation. Our objective was to delineate how CHCs in the feces and on the cockroach body operate in conditions that reflect the German cockroach's ecology—either applied to shelters, representing fecal deposition, or to previously extracted cockroaches, representing shelter co-habitation with other cockroaches. Cockroaches and feces-conditioned filter papers were extracted, CHCs were purified by flash chromatography, and two-choice behavior assays were performed with first instar nymphs. Our results confirmed that nymphs preferred to rest within feces-conditioned shelters. However, purified CHCs did not elicit more aggregation than solvent-treated control shelters. Nymphs significantly preferred to rest in shelters that contained a CHC-free dead female, but the addition of CHCs to the female did not enhance aggregation. Nymphs preferred to aggregate with the CHC-free female over CHC-treated shelters. Finally, a methanol extract of feces was highly effective at eliciting aggregation, contesting previous reports that fecal CHCs serve as aggregation pheromone. We assert that CHCs play a minor, if any, role in the aggregation behavior of German cockroaches.

Key words: hydrocarbons, aggregation, pheromone

Introduction

Many species of insects form active aggregations, resting aggregations, or both. Active aggregations generally coincide with feeding periods and may serve to overcome the host, protect the aggregates from predators and parasitoids, or thermo- and hygro-regulate (Parrish et al. 1997). Aphids (*Aphis varians* Patch), for example, aggregate on substrates to decrease the risk of predation (Turchin and Kareiva 1989), bark beetles that attack healthy trees aggregate to overcome tree defenses (Gitau et al. 2013), and necrophilous maggots thermoregulate in mass aggregations to accelerate their development (Aubernon et al. 2016). Resting aggregations are usually formed during the inactive periods in places presenting environmental conditions that fulfill the ecological requirements of the species (Wertheim et al. 2005). For example, choice of rest sites can be guided by microclimatic factors such as temperature and humidity, and physical features such as size, color, and texture of the substrate. In both types of aggregations, environmental and social cues orient insects toward each other, focusing them to gather at a preferred site (Wertheim et al. 2005, Imen et al. 2015). In insects, group formation is often guided and sustained by aggregation pheromones, which may attract conspecifics to preferred sites and cause arrestment at the site (Wertheim et al. 2005, Imen et al. 2015).

Most cockroach species are nocturnal and aggregate in communal shelters during the day (Schal et al. 1984, Bell et al. 2007). Many factors affect aggregation behavior in the German cockroach, *Blattella germanica* L., including preferences for certain sized shelters (Berthold and Wilson 1967), texture and vertical vs. horizontal orientation (Bell et al. 1972), presence of conspecifics (Koehler et al. 1994), aggregation pheromones (Ishii and Kuwahara 1967), and other factors (e.g. Rust et al. 1995). A variety of chemicals have been proposed as aggregation pheromones of the German cockroach, including rectal pad secretions (Ishii and Kuwahara 1967), short- and medium-chain volatile fatty acids in feces (Ritter and Persoons 1974, McFarlane and Alli 1986, Wada-Katsumata et al. 2015), alkylamines and blattellastanoside A and B (Sakuma and Fukami 1990, 1993), and cuticular hydrocarbons (Rivault et al. 1998).

Cuticular hydrocarbons (CHCs) comprise a thin apolar layer on the outer surface of insects. They have many functions, including as a protective barrier from microorganisms and water loss (Howard and Blomquist 2005, Blomquist and Bagnères 2010). Hydrocarbons also play important roles as semiochemicals, including as nestmate and caste recognition pheromones in social insects (Lahav et al. 1999, van Zweden and d'Ettorre 2010, Funaro et al. 2018), as sex pheromones and pheromone precursors (Ferveur 2005, Jurenka et al. 2016), and as aggregation pheromones in some insects. For example, (*Z*)-10-heneicosene was found to be attractive to both male and female *Drosophila virilis* Sturtevant and considered to be part of their aggregation pheromone (Bartelt and Jackson 1984).

Cuticular hydrocarbons are naturally found on the cuticle and in the feces of *B. germanica* which contaminate their shelters, and they have been implicated as an aggregation pheromone (Rivault et al. 1998, Lihoreau et al. 2012). Recent studies of cockroach aggregation pheromones have confounded substrate-deposited chemicals (e.g., feces-produced) that do not require the presence of a cockroach, with cues that stimulate joining a cockroach or a group. In this study, we sought to determine the function of CHCs on the shelter (contaminated with compounds from feces and the body) and on the cockroach body, and investigate the relationship between CHCs and other aggregation cues. We focused on filter paper shelters because in a previous report CHCs on shelters elicited aggregation of nymphs (Rivault et al.

1998). Because CHC signals can also emanate from the body surface of conspecifics, we also assessed the effectiveness of CHCs when applied to pre-extracted insects. Therefore, we separated our queries about the roles of CHCs in aggregation behavior into two ecological contexts: a single nymph given a choice of two variously treated shelters, or a nymph given a choice to shelter with a treated female within a treated shelter. In our experiments, other features of the shelters (e.g., darkness, texture) were held constant. We sought to re-assess the behavioral activity of CHCs as an aggregation pheromone in the German cockroach by testing CHCs and other chemicals as aggregation agents. Based on recent findings (Wada-Katsumata et al. 2015), we hypothesized that polar lipids might play more prominent roles in guiding aggregation behavior than CHCs. Behavioral experiments confirmed that German cockroach nymphs preferred to shelter under feces-contaminated shelters, but CHCs did not elicit aggregation behavior. Conversely, more polar lipids from conditioned filter papers were significantly more effective at stimulating aggregation than CHCs. Understanding aggregation behavior in cockroaches can inform innovative pest management strategies for mitigating their adverse health effects in the indoor environment.

Materials and Methods

Cockroaches

The insects used in these experiments were from a laboratory strain of *B. germanica* (Orlando Normal, collected in a Florida apartment over 70 years ago). They were reared on food pellets (Purina No. 5001 Rodent Diet, PMI Nutrition International, St. Louis, MO) and water in cotton-stoppered vials at 27 ± 1 °C, 40–70% relative humidity, and L:D = 12:12 photoperiod. Adult females were virgin of known ages, as indicated below, and first instars used in behavioral assays were ~1 day after hatching.

Female-Conditioned Filter Papers

Conditioned filter papers were prepared by allowing four groups of 20 adult females (0–1 days old) to condition (touch and defecate upon) filter papers for seven days, representing the vitellogenic period when food consumption is high (Schal et al. 1997). Each petri dish (14 cm diameter) contained 20 females, rodent chow, water in a cotton-stoppered glass vial, and an accordion-folded filter paper (Whatman #1, 24 cm, Pittsburgh, PA).

Extraction and Fractionation of Female-Conditioned Filter Papers

After conditioning, the filter papers were immersed in 10 ml of chloroform in 20 ml glass vials, vortexed intermittently for 5 min, and the chloroform was transferred to a clean vial. This process was repeated two more times and the chloroform extracts were combined. The extracts were reduced to approximately 0.1 ml using a gentle stream of high-purity nitrogen. The filter paper extract was then separated into two fractions: CHCs and polar lipids. The extract was loaded onto a dry silica gel (70-230 mesh, EM Science, Gibbstown, NJ) column (Pasteur pipette loaded with 0.5 g silica gel, washed with dichloromethane, dried with nitrogen, activated 60 min at 110°C) and dried under a stream of nitrogen. The walls of the vial were washed twice with 0.5 ml of chloroform, reduced to approximately 0.1 ml, and loaded onto the column. The silica gel was then dried with a stream of nitrogen. The column was first eluted with 5 ml hexane into a glass vial to obtain CHCs (hexane fraction). Then the column was eluted with 5 ml ethyl acetate and 5 ml methanol which were combined in a glass vial to obtain more polar lipids. A total of 4 columns were used during the experiment (one for each conditioned filter paper), yielding 4 vials for each fraction. Each fraction was reduced to about 0.2 ml and all four hexane fractions transferred to the same 1.5 ml vial and all four polar fractions were transferred to another 1.5 ml vial. The walls of the vials were washed twice with clean hexane (CHC fractions) or chloroform (polar fractions) and the washes were combined in the respective 1.5 ml vial. The solvent was reduced to near dryness and 1 ml of hexane or chloroform was added so each vial contained 80 females-7 day-equivalents (FEs) in 1 ml of solvent, or 12.5 µl per FE.

Additionally, four new conditioned filter papers were prepared using 80 adult females (0–1 days old, 20 females per filter paper), as described above. These filter papers were extracted sequentially using three different solvents: chloroform, methanol, and water. Each filter paper was first immersed in 10 ml of chloroform in a 20 ml glass vial, then vortexed twice for 2 min each. The filter paper was removed and allowed to dry under the hood overnight. The chloroform was removed and transferred to a new glass vial. The walls of the vials were washed twice and added to the 20 ml vial. The four extracts were blown down to ~2 ml and combined into one vial. The combined extract was then blown down with nitrogen to 1 ml. This extraction process was repeated using methanol, and the filter paper was again allowed to dry overnight. The conditioned filter papers were finally extracted with water and the extract was reduced using a vacuum concentrator (Jouan RCT 60, Thermo Scientific, Waltham, MA). All the extracts were kept in a -30°C freezer for behavior assays.

Extraction of Cockroaches

We extracted females because they contain more CHCs than any other life stage (Jurenka et al. 1989), and females spend more time in association with first instar nymphs (Lihoreau et al. 2012). Fourteen groups of 20 adult females each (5–6 days old) were placed in 20 ml glass vials and frozen at -30°C. Each vial was then allowed to thaw to room temperature. The insects were covered in hexane (~10 ml), gently swirled for 2 min, and the hexane was transferred to a clean glass vial. This process was repeated twice, and the hexane extracts were combined. The hexane extract and extracted females were kept in a -30° freezer for behavior assays. To purify CHCs, the extract was applied to a silica gel column, as described above, CHCs were eluted with 5 ml hexane, the solvent reduced to near dryness and 2.8 ml of hexane was added to the vial, yielding 280 FEs of CHCs in 2.8 ml, or 1 FE per 10 µl.

Quantification of Cuticular Hydrocarbons

Aliquots of extracted filter papers, extracted females, and their respective CHC fractions were analyzed by gas chromatography. An internal standard (*n*-hexacosane = *n*-C26, 10 µg) was added to each vial, the solvent was evaporated to dryness and the residue taken up in 100 µL hexane in a glass insert within a 1.5 mL GC autoinjection vial. One of 100 µL was injected in pulsed splitless mode using a 7683B Agilent autosampler into a DB-5 column (20 m x 0.18 mm internal diameter x 0.18 µm film thickness, J&W Scientific, Folsom, CA) in an Agilent 7890 series GC (Agilent Technologies, Santa Clara, CA) connected to a flame ionization detector (FID) with ultra-high purity hydrogen as carrier gas (0.75 mL/min constant flow rate). The inlet was held at 300℃, FID at 320℃, and the column was held at 50°C for 1 min, increased to 320°C at

10°C/min, and held at 320°C for 10 min. Total peak area was used for the calculation of total CHC amount.

Behavior Tests

Binary-choice bioassays were conducted in disposable 60 x 15 mm plastic petri dishes (Falcon-Corning, Corning, NY) using individual first instar nymphs to avoid signaling among nymphs. A single nymph was placed between two folded tent-shaped filter paper shelters, each 2 x 2 cm (4 cm^2) (Fig. 1A,B). Unless otherwise indicated, one shelter was either a conditioned filter paper (positive control) or treated with an extract, and the other shelter was treated with the respective solvent only. The assays were conducted at 27°C and under the same 12:12 hr L:D cycle as rearing conditions. Fluorescent lights were on during the photophase, when cockroaches tend to rest within shelters, and a red headlight was used to observe the assays during the scotophase. The assays were set up late in the photophase, and the position of each nymph within the petri dish was recorded 24 hrs later (in the photophase), allowing insects 12 hrs of nighttime activity before settling to shelter for the day. Control assays were completed to evaluate any directional bias in the assay conditions by giving individual nymphs a choice between two hexane-treated shelters.

In other assays, solvent-extracted females were placed inside a shelter to determine if they could guide nymphs to choose specific treatments. Females that had been extracted with hexane were extracted again in chloroform for 5 min, the chloroform extract was discarded, and the extracted females were allowed to dry overnight in a fume hood. This step was repeated in order to ensure there were no attractive substances on the extracted female body.

In these aggregation assays, we tested various combinations of an extracted (CHC-free) female placed under a shelter, with either the female or the shelter treated with various extracts. The treatments were (a) CHC-free female in a hexane-treated shelter vs. hexane-treated shelter; (b) CHC-free female in a hexane-treated shelter vs. CHC-treated shelter; (c) CHC-free female in a hexane-treated shelter vs. CHC-free female treated with CHCs in a hexane-treated shelter; and (d) CHC-free female in a CHC-treated shelter vs. CHC-free female treated with CHCs in a hexane-treated shelter.

In assays of sequentially extracted conditioned filter papers, nymphs were given a choice between a shelter treated with each respective extract and another shelter treated with the corresponding solvent. The three extracts were then recombined, and behavior assays were conducted between a filter paper shelter treated with the combined extracts and a filter paper shelter treated with the three solvents (chloroform, methanol, and water).

Statistics

Differences in aggregation between the two filter paper shelters were tested using a Chi-square test of independence ($\alpha = 0.05$) performed in Microsoft Excel.

Results

Cuticular Hydrocarbons on Females and Female-Conditioned Filter papers

We recovered 159.8 ± 8.57 µg total CHCs per female cockroach ($n = 6$). Therefore, 1 FE of female extract or CHC-fraction applied to a filter paper shelter represented 40 µg/cm^2. We recovered less CHCs from filter papers conditioned for 7 days by 20 adult females (3.24 ± 7.38

µg per female), so 1 FE of conditioned filter paper extract applied to a shelter represented 0.81 µg/cm^2.

Aggregation in Female-Conditioned Shelters

First instar nymphs were used in behavior assays (Fig. 1A,B) because they are highly motivated to aggregate during the photophase (Ishii and Kuwahara 1967). Moreover, we used a single nymph in each assay to preclude interactions among nymphs that may result in some nymphs following early responders. First instar nymphs sheltered equally under each of two hexane-treated filter paper shelters (Chi-square test, $\chi^2 = 1.0869$, $p = 0.2971$, $n = 26$ (Fig. 1C), indicating that our environmental assay conditions did not provide nymphs any inadvertent directional cues. In four replicate assays, the nymph remained outside both shelters. Nymphs significantly preferred to rest under filter paper shelters conditioned (defecated upon) by adult cockroaches ($\chi^2 = 29$, $p < 0.0001$, $n = 29$) (Fig. 1D), indicating that feces and other secretions from adult females contained a highly effective aggregation pheromone.

Aggregation in Shelters Treated with Extracts and Fractions

Cockroach CHCs can guide conspecific aggregation behavior under two distinct ecological contexts – as components of feces and as components of the cuticular lipids of conspecifics. In two-choice assays, first instar nymphs sheltered slightly more under hexane-treated shelters than under shelters treated with 1 FE, or 159.8 µg, of CHCs (40 µg/cm^2) purified from the female cockroach body, although there was no significant difference between these two choices ($\chi^2 = 1.96$, $p = 0.1615$, $n = 25$) (Fig. 1E). This suggested that CHCs, which are found at a lower concentration in the feces, would fail to elicit aggregation alone.

We then tested whether more polar lipids extracted from conditioned filter papers would provide better stimuli for aggregation behavior than CHCs. We first examined fractions of chloroform-extracted conditioned filter papers. First instar nymphs did not show a significant preference for shelters treated with either 1 FE of the CHC fraction or of the more polar fraction ($\chi^2 = 0.8$, $p = 0.3711$, $n = 20$), with the nymph in 10 of 30 replicate assays not resting in either shelter (Fig. 1F). Finally, we sequentially extracted the conditioned filter papers in three solvents of increasing polarity: first with chloroform, then methanol, and lastly with water. First instar nymphs significantly preferred to rest within shelters treated with 1 FE of the methanol extract over shelters treated with methanol only ($\chi^2 = 21.16$, $p < 0.0001$, $n = 25$) (Fig. 2B). They showed no significant preferences between shelters treated with either 1 FE of the chloroform or water extracts and shelters treated with the respective solvent controls, chloroform ($\chi^2 = 0.3333$, $p = 0.5637$, $n = 27$) (Fig. 2A) and water ($\chi^2 = 1.5$, $p = 0.2207$, $n = 25$) (Fig. 2C). First instar nymphs also significantly preferred 1 FE of the re-combined extract of chloroform, methanol, and water over the combined chloroform, methanol, and water solvent-treated shelter ($\chi^2 = 13.37$, $p = 0.0003$, $n = 27$) (Fig. 2D).

Aggregation in Shelters Occupied by Conspecific Females

We tested whether aggregation responses could be influenced by CHCs on the surface of a cockroach within the shelter. The presence of a dead female within the shelter guided nymphs to that shelter. First instar nymphs sheltered significantly more in a shelter containing a thoroughly solvent-extracted CHC-free female cockroach than under either a hexane-treated shelter with no cockroach ($\chi^2 = 12.45$, $p < 0.0001$, $n = 29$) (Fig. 3A) or a shelter treated with 1 FE of CHCs (159.8 µg, or 40 µg/cm^2) with no cockroach ($\chi^2 = 4.4815$, $p = 0.0343$, $n = 28$) (Fig. 3B). These results showed that the presence of a CHC-free cockroach alone could be a stronger aggregation stimulus than CHCs applied to the shelter.

To further investigate the role of female-associated CHCs, a solvent-extracted female was treated with 1 FE of CHCs and placed under a hexane-treated shelter and compared to an extracted (CHC-free) female treated with hexane only under a hexane-treated shelter. There was no significant difference in the aggregation preference of the first instar nymphs ($\chi^2 = 0.1429$, $p = 0.7055$, $n = 29$) (Fig. 4A). Moreover, direct comparisons of the two ecological contexts in which CHCs could operate revealed that first instar nymphs slightly, but not significantly, preferred to shelter with the CHC-treated female within a hexane-treated shelter over a CHC-free female within a CHC-treated shelter ($\chi^2 = 2.2857$, $p = 0.1306$, $n = 28$) (Fig. 4B). Thus, CHCs associated with both conspecifics and with the shelter were ineffective at guiding aggregation of individual cockroaches.

Discussion

A variety of cues and signals can guide the decision of cockroaches to seek shelter, where to shelter and with whom to aggregate. The aggregation of cockroaches in shelters is considered a self-organizing process (Deneubourg et al. 2002), where for an individual cockroach, the larger the number of sheltering neighbors, the more likely the individual is to stop and stay beside them (Garnier et al. 2009). Therefore, an individual cockroach is more likely to choose to aggregate under a shelter where other cockroaches are already present, and the decision to join a group has a density-dependent element. This tendency for collective and density-based decision-making can challenge studies of the proximate mechanisms that underlie aggregation behavior because of the inherent difficulties of separating cues that guide individual decisions from those that guide the group. Although individual nymphs and groups of nymphs may exhibit similar preferences in simple aggregation assays with conditioned shelters (e.g., Rivault and Cloarec 1998), the group may compromise our ability to assess the relative contributions of various cues and signals to this preference. Therefore, in this study we used individual nymphs, rather than groups of nymphs, to eliminate three related limitations of group assays: (a) collective decisions by a group may violate assumptions of independence of individual behavioral choices and statistical tests; (b) the presence of a group can alter the characteristics of the shelter, especially when shelter features are suboptimal (e.g., too light, too large); and (c) groups of nymphs are much more likely to defecate and thus alter the quality of the shelter during long-duration assays (e.g., 24 hrs), which are common in such studies. The tendency of cockroaches to aggregate together as a group is clearly documented in studies – all members of a group tend to shelter together on one of two equally suitable shelters (e.g., Ame et al. 2004, Said et al. 2005), making it impossible to disentangle individual responses to shelter-associated cues (e.g., fecal pheromone) from group-associated cues. This is particularly evident when shelter characteristics are inadequate for cockroach aggregation. Individual cockroaches may ignore poor quality shelters, but they accept the same shelter as a group (e.g., Sempo et al. 2009), likely because the group itself altered the shelter characteristics (e.g., darker, more shadows, more crevices).

Signals and Cues Associated with Shelters

In two-choice behavior assays with individual first instar nymphs, the nymphs significantly preferred shelters conditioned by adult females over unconditioned shelters, indicating that fecal, oral and/or body secretions on the conditioned shelters, possibly including CHCs, serve as aggregation pheromone. Surprisingly however, nymphs rested equally in hexane-treated shelters as in shelters treated with chromatographically purified female CHCs. To compare the CHC fraction to more polar lipids, we extracted conditioned filter papers in chloroform and assayed nymphs with the CHC-fraction and a polar fraction obtained from flash chromatography. Nymphs again rested equally in both shelters, and ~33% of first instar nymphs remained outside both shelters. These findings indicated that (a) CHCs were ineffective as an aggregation pheromone, and (b) chloroform failed to extract behaviorally active chemicals from conditioned filter papers.

The lack of behavioral activity in chloroform extracts of conditioned shelters suggested that more polar compounds should be considered. Therefore, we sequentially extracted female-conditioned filter papers with solvents of increasing polarity: chloroform, methanol and water. Consistent with our previous assays, nymphs exhibited no preference for shelters treated with either the chloroform extract or the water extract versus shelters treated with the respective solvent. However, nymphs significantly preferred shelters treated with methanol extracts over shelters treated with methanol only, and shelters treated with the recombined three-solvent extracts also were significantly preferred over the solvent-treated shelters. These results suggested that polar lipids are more important than CHCs in inducing German cockroach aggregation, and these compounds were excluded from our hexane and chloroform extractions.

The literature on the role of CHCs in shelter choice by cockroaches is fragmentary and often contradictory. For example, American cockroach (*Periplaneta americana* L.) nymphs were shown not to aggregate any more on cuticular dichloromethane extract-treated shelters than on solvent only (dichloromethane) treated shelters (Said et al. 2005, Imen et al. 2015), but other dichloromethane extracts were used successfully to guide cockroaches to shelter with robots (Halloy et al. 2007). Several factors have contributed to these disparities. First and foremost, when insects are extracted, CHCs are often not separated from crude extracts of cuticular lipids, precluding a direct attribution of the behavior to CHCs because cuticular extracts may contain triglycerides, fatty acids, sterols and other lipids, some of which effectively stimulate aggregation (McFarlane and Alli 1986, Scherkenbeck et al. 1999, Wada-Katsumata et al. 2015). Second, because compounds excreted in feces are often adsorbed to cuticular lipids (Ishii and Kuwahara 1967), behavioral responses to shelters treated with high doses of crude cuticular extracts (e.g., Imen et al. 2015) may be confounded by fecal compounds. Third, methodologies related to chemicals (e.g., extraction, fractionation, concentration) and assay procedures (e.g., individual vs. group, photophase vs. scotophase) vary considerably among studies. Because we aimed to investigate the role of non-volatile CHCs, we allowed solvent extracts and fractions to evaporate to dryness, so it is possible that volatile compounds that may be important for aggregation were lost during these procedures. Notably, volatile carboxylic acids extracted from feces with methanol were highly effective aggregation cues in both olfactometer and sheltering assays (Wada-Katsumata et al. 2015). Finally, when groups of live insects are used in binary assays, it is impossible to separate the effects of CHCs and other semiochemicals from their feces that may differ because their gut microbiomes may differ (Wada-Katsumata et al. 2015, Kakumanu et al. 2018).

Signals and Cues Associated with Conspecifics

Our conclusion that CHCs from conditioned filter papers do not serve as an aggregation pheromone was confirmed in a second ecological context (CHCs on the cockroach body surface) using the CHCs of adult females placed either on shelters or on extracted CHC-free females. The premise of this assay paradigm was that CHCs on conspecifics within a shelter might bias the nymph's choice toward this aggregation site or the conspecific. In our assays, nymphs significantly preferred to rest under a shelter that contained a thoroughly extracted CHC-free female. They also preferred a CHC-free female over a female-free shelter treated with female CHCs. Furthermore, the addition of CHCs either to an extracted female within a shelter or to the other shelter containing a CHC-free female did not contribute to shelter preferences by nymphs. Overall, these results with females and their CHCs indicate that CHCs do not influence or bias the nymphs' preferences in two-choice assays.

Assay designs with a female in one shelter and not in the other shelter are inherently asymmetrical, and therefore difficult to interpret. Although it is possible that species-specific non-chemical signals from the solvent-extracted female guided the nymphs to shelter with her, we suspect that the most prominent cues that guided nymphs to the dead female were positive thigmotaxis and negative phototaxis. Cockroaches prefer to rest in dark, tight-fitting crevices that provide tactile stimuli to their ventral and dorsal surfaces (Berthold and Wilson 1967). The extracted female likely fragmented and reduced the open space within the shelter, darkened it, and provided the thigmotactic stimuli that nymphs seek. A similar confounding influence of thigmotaxis and phototaxis may be evident in aggregation tests using cockroach extract-treated robots. In two-choice aggregation assays in a large arena, *P. americana* preferred to assemble with extract-treated robots under a lighter, less attractive shelter than in a darker but empty shelter (Halloy et al. 2007). It is possible, however, that the robots simply improved the quality of the inadequate shelters in at least two ways unrelated to the extract or robot behavior. Moreover, because crude cuticular extracts were used to make robots more attractive, it is possible that non-CHC components of the extract played some role since cuticular extracts of *P. americana*, like other insects, contain some triglycerides, free fatty acids, sterols and other lipids (Jackson 1972). These experiments, reported by Halloy et al. (2007), could not resolve the influence of multiple cues upon cockroach preferences. Our results with asymmetrical assays indicated that CHCs did not change the preference of *B. germanica* nymphs, suggesting that these compounds do not take precedence over other signals and cues in the cockroach's environment.

In symmetrical aggregation assays with *P. americana*, adults preferred to assemble with a cuticular lipid extract-treated robot under a shelter rather than in shelters containing a solvent-treated robot (Halloy et al. 2007). We performed a similar assay with *B. germanica* in this study, but with a solvent-extracted dead female rather than a robot. A nymph was given a choice to shelter with an extracted CHC-free female treated with CHCs under a hexane-treated shelter versus an extracted female treated with solvent only under a hexane-treated shelter. Nymphs exhibited no significant aggregation preference for either CHC-treated or CHC-free females. It is possible that this apparent discrepancy is related to species-specific differences (*P. americana* vs. *B. germanica*), or to methodological differences such as the purity of CHCs, amount of CHCs used (both studies treated the substrate the same concentration per cm^2 as found on the respective cockroach cuticle), and the substrate on which CHCs were placed. Future investigations should conduct comparative studies using a standard methodology.

Aggregation Pheromone of *B. germanica*

The chemical identification of aggregation pheromone in *B. germanica*, and the role of CHCs as aggregation pheromone, have been contentious since the early documentation of aggregation in this species. Ishii and Kuwahara (1967) observed that ether and methanol extracts of cockroaches and cockroach-conditioned papers elicited aggregation, but they concluded that the aggregation pheromone was associated with feces that contaminated the cuticular lipids of cockroaches. A series of investigations by multiple labs followed, most showing that the active compounds were fatty acids associated with feces (Ishii and Kuwahara 1967, Ritter and Persoons 1974, Scherkenbeck et al. 1999, Wada-Katsumata et al. 2015). Other compounds were also identified as attractants (volatile alkylamines) and arrestants (glycosylated steroids: blattellastanoside A and B) (Sakuma and Fukami 1990, 1991), but the behavioral activity of the latter has been contested (Scherkenbeck et al. 1999).

A series of reports, starting with Rivault et al. (1998), challenged the notion that polar lipids are involved, and instead concluded that CHCs comprise the aggregation pheromone of *B. germanica*. Unlike subsequent investigations by this team, however, Rivault et al. (1998) was the only study that separated CHCs from other cuticular lipids; therefore, this report deserves particular scrutiny. Using two-choice assays with groups of 20 first instar nymphs and filter paper substrates treated with various extracts, Rivault et al. (1998) found that dichloromethane and pentane extractions of cockroaches were most effective at inducing aggregation, while methanol extracts failed to do so. However, CHCs purified by flash chromatography from dichloromethane extracts failed to elicit aggregation. Rivault et al. (1998) nevertheless speculated that some CHCs were lost during the fractionation procedure, which eliminated their effectiveness. But several observations suggest otherwise. First, because *B. germanica* CHCs range from C27 (MW 379) to C32 alkanes and mono- and dimethyl alkanes (MW 463), they are essentially non-volatile and easily recovered almost quantitatively from flash chromatography columns. Second, although methanol extraction is not an efficient method for recovering CHCs, the chromatograms in Rivault et al. (1998) show that the methanol extracts contained similar amounts of CHCs (with several linear and near terminally branched alkanes less represented), yet this fraction was inactive. Third, each filter paper substrate was treated with the extract of 15 sixth instar nymphs, and this amount far exceeds the amount of CHCs found in feces. Fourth, careful bioassay-guided fractionations have not found behavioral activity in the CHC fraction (Scherkenbeck et al. 1999). Fifth, there is ample evidence from other species of cockroaches (Jackson 1972) and from *B. germanica* (Schal, personal observation) that their cuticular surface contains fatty acids and other polar lipids, in addition to CHCs. Sixth, methanol extracts have proven effective in various olfactometer assays (Sakuma and Fukami 1985, Wendler and Vlatten 1993, Sakuma et al. 1997, Wada-Katsumata et al. 2015). Lastly, our results showed that CHCs, purified from either feces-contaminated filter papers or adult females, were ineffective at eliciting aggregation in *B. germanica*. We confirmed that CHCs were not effective at eliciting aggregation behavior by showing that purified CHCs were ineffective, CHCs applied to extracted CHC-free females did not increase her attractiveness to nymphs, and chloroform extracts that contain CHCs were ineffective.

In our assays, methanol extractions of feces-contaminated papers recovered a highly effective aggregation pheromone, as in previous studies (Kitamura et al. 1974, Wendler and Vlatten 1993, Miller et al. 1997, Scherkenbeck et al. 1999, Wada-Katsumata et al. 2015). We conclude that CHCs play a minor, if any, role in the aggregation behavior of the German cockroach. It is likely that the aggregation pheromone consists of attractants that guide the early stages of the aggregation process and arrestants that act through contact (Mori and Fukamatsu 1993, Sakuma and Fukami 1993, Rivault and Cloarec 1998). Feces-associated attractants, mainly carboxylic acids and amines, some of which may be microbial products, clearly play prominent roles as aggregation pheromone components. However, their importance relative to other chemicals, as well as to tactile (Lihoreau and Rivault 2008, Uzsák et al. 2014) and acoustic signals (Mistal et al. 2000), remains unknown. Nevertheless, in this study we showed that CHCs associated with either conspecifics or with the shelter were ineffective alone at guiding aggregation.

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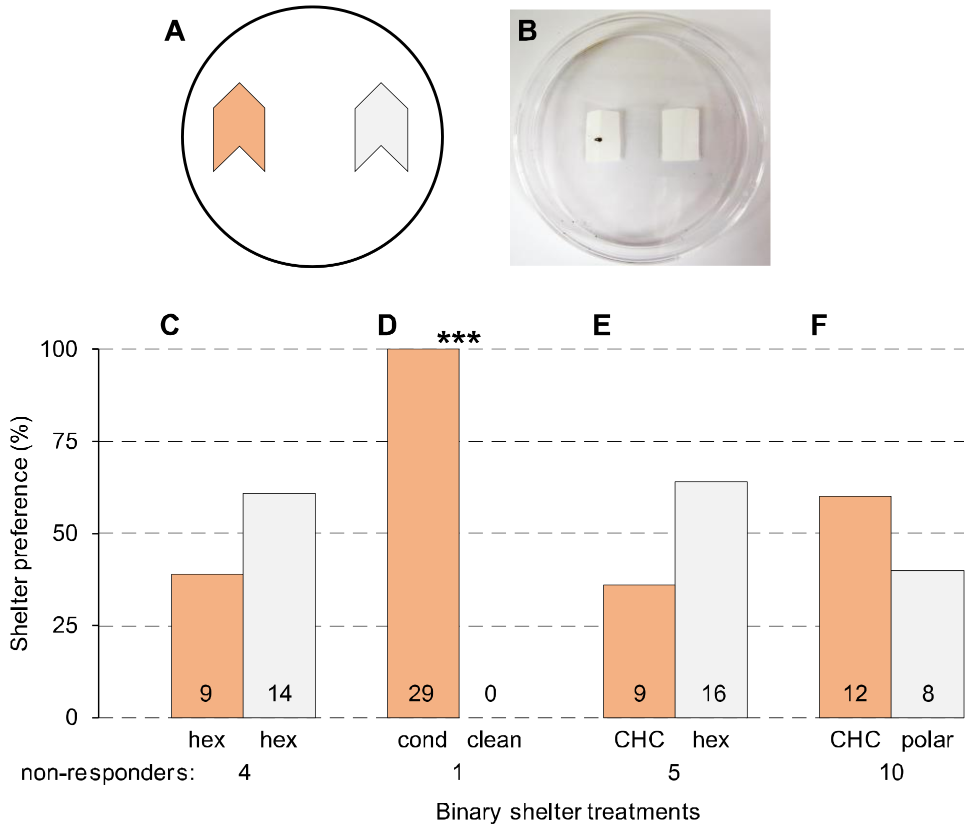

Figure 1. Binary behavioral assay design and aggregation preferences of first instar nymphs under filter paper shelters in two-choice assays. Individual first instar nymphs were tested in Petri dish assays (A, B) with two filter paper shelters treated with the specified materials. Treatments included hexane vs. hexane (C), female-conditioned shelters vs. clean shelters (D), CHCs obtained from conditioned shelters vs. hexane (E), and CHCs obtained from conditioned shelters vs. polar lipids obtained from conditioned shelters (F). Shelter choices (%) are shown, along with number of assays with individual nymphs resulting in preference for each shelter, and number of assays resulting in nymphs resting outside either shelter. Assays with *** indicate $p < 0.001$; in other assays $p > 0.05$.

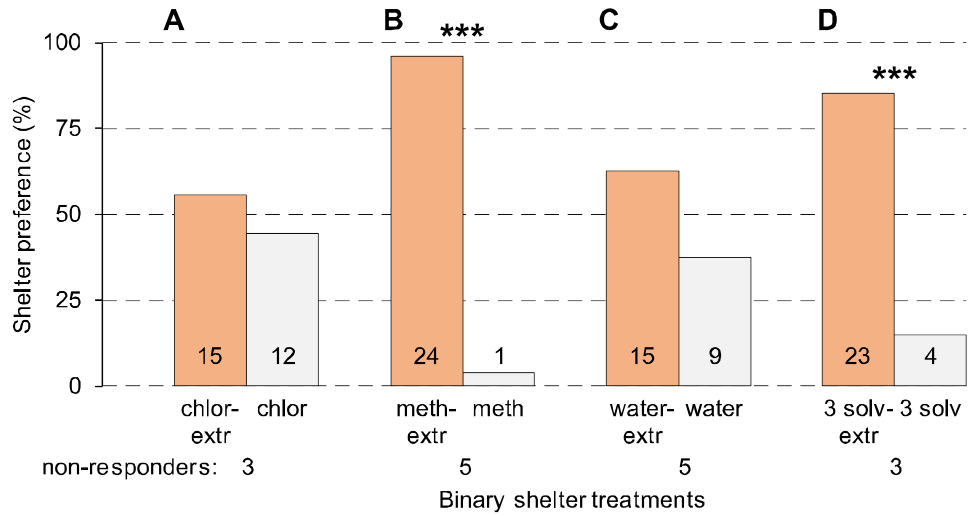

Figure 2. Recovery of aggregation preference with solvent extraction of adult-conditioned paper. Individual first instar nymphs were tested in two-choice assays with filter paper shelters treated with various solvent extractions of female-conditioned filter papers. Conditioned papers were extracted sequentially with chloroform (chlor-ext) (A), methanol (meth-extr) (B) and water (water-extr) (C), the extracts were applied to filter paper shelters, and paired with shelters treated with the respective solvent as control. In (D), the three extracts were recombined (3 solv-extr) and assayed against the combined solvents (3 solv). Shelter choices (%) are shown, along with number of assays with individual nymphs resulting in preference for each shelter, and number of assays resulting in nymphs resting outside either shelter. Assays with *** indicate $p < 0.001$; in other assays $p > 0.05$.

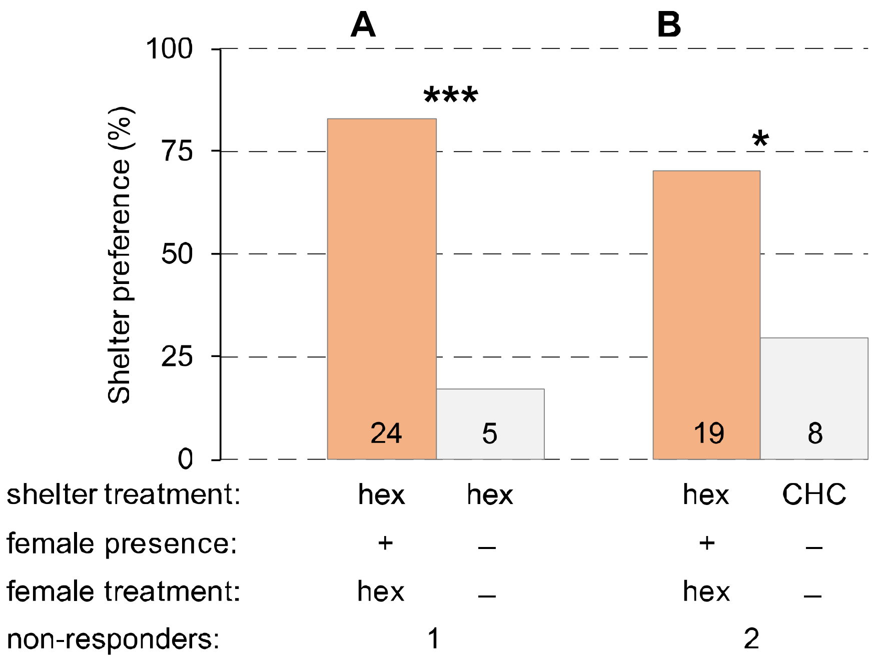

Figure 3. Effect of presence of an extracted female within a shelter on aggregation preference of first instar nymphs in two-choice assays. Individual first instar nymphs were tested in assays with filter papers treated with female CHCs or hexane (hex) only. In (A), both shelters were treated with hexane, and a thoroughly extracted dead female was treated with hexane and placed in one of the shelters. In (B), one of the shelters was treated with female CHCs and the other shelter received an extracted female. Shelter choices (%) are shown, along with number of assays with individual nymphs resulting in preference for each shelter, and number of assays resulting in nymphs resting outside either shelter. Assays with *** indicate $p < 0.001$, * indicates $p < 0.05$.

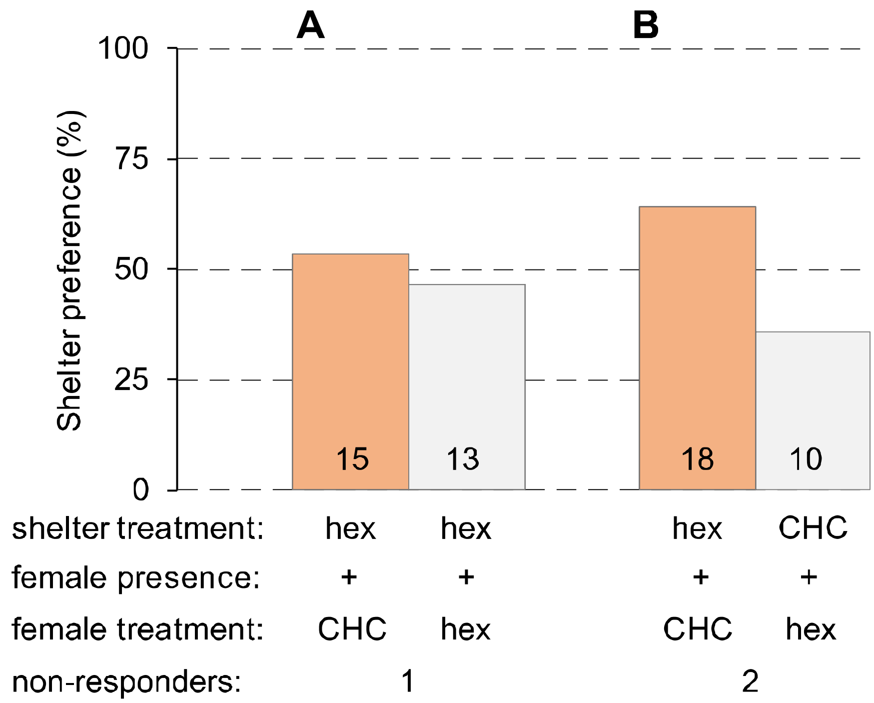

Figure 4. Effect of presence of a CHC-treated female within a shelter on aggregation preference of first instar nymphs in two-choice assays. Individual first instar nymphs were tested in assays with filter papers treated with female CHCs or hexane (hex) only. In (A), both shelters were treated with hexane, and a thoroughly extracted dead female was placed in each shelter. One female was treated with hexane and the other with female CHCs. In (B), one of the shelters was treated with female CHCs and the other with hexane. Both shelters received an extracted female, but the female in the hexane-treated shelter was also treated with female CHCs. Shelter choices (%) are shown, along with number of assays of individual nymphs resulting in preference for each shelter, and number of assays resulting in nymphs resting outside either shelter. In both sets of assays $p > 0.05$.

CHAPTER 3

The Effects of Diet and Feces Quality on German Cockroach Development, Aggregation Behavior, and Colony Recognition

Abstract

Microbial communities colonize the insect gut and regulate various aspects of the insect's biology, including behavior. In cockroaches, individuals within colonies engage in various behaviors, such as food-sharing and coprophagy that promote distribution of gut microbes within the colony. Because aggregation behavior is mediated by volatile organic compounds associated with fecal microbes, members of an aggregation are also expected to share cues that guide aggregation behavior. The chemical identities of these aggregation pheromones remain unclear, possibly because different colonies host different microbial assemblages that produce different metabolites. In this study, we experimentally manipulated the gut microbiomes of sterile neonate cockroaches with different fecal inocula and various balanced diets and assessed their aggregation preferences in choice bioassays. Our results demonstrated that nymphs significantly preferred aqueous extracts of their own colony feces over fecal extracts from colonies that fed on a different diet. These findings suggest that unique gut microbial communities in each cockroach colony produce colony-specific aggregation signals that guide colony fidelity to specific shelters. Cockroach colonies fed the same diet lost their ability to discriminate their own colony odor from other colonies, even when they were inoculated as neonates with different colony-specific microbes produced by cockroaches on different diets. Diet quality also significantly affected cockroach development, but the presence of conspecific feces had no discernible additional benefits. We conclude that diet shapes the microbial community of the cockroach gut, which in turn produces a unique aggregation pheromone blend, which is deposited in feces and guides colony recognition. This colony

recognition system, using microbial metabolites, is functionally similar to the use cuticular hydrocarbons and other lipids in nestmate recognition in social insects.

Key words: feces, cockroach, coprophagy, aggregation, colony recognition

Introduction

Communication and sharing of information between insects often occur through the use of chemical messages known as semiochemicals that can be divided into two categories: allelochemicals (interspecies signals) and pheromones (intraspecies signals). Pheromones are secreted by individuals and are detected and decoded by conspecifics, guiding their behavior (releaser pheromones) or development and physiology (primer pheromones) (Wyatt 2014). The pheromonal message can inform conspecifics about the presence of danger (dispersal or alarm pheromones), location of a resource (recruitment or trail pheromones), presence and quality of a mate (sex pheromones), or presence of a group to join (aggregation pheromones).

Many non-social insects depend on aggregation pheromones as the major means of bringing individuals together for more efficient foraging, protection from environmental stressors and predators, and other adaptive benefits that are accrued in foraging or resting in groups (Ali and Morgan 1990). Sex pheromones are species-specific and are often emitted at certain times to avoid similar pheromone blends of closely related species. In contrast, aggregation pheromones may be highly species-specific, but they often operate across species boundaries. For example, different species of earwigs are attracted to both trails and shelters marked by other species and it was hypothesized that the aggregation pheromone of the best-represented species could act as a kairomone for cohabiting individuals of closely-related species (Sauphanor and Sureau 1993). Multiple studies with different cockroach species have shown, for example, that nymphs aggregate on filter papers conditioned by fecal contamination of either their own or other species (Ishii 1970, Bell et al. 1972, Roth and Cohen 1973, Rust and

Appel 1985). The mechanisms that underlie the broad overlap in aggregation signals among species are not known.

In many insects fecal components guide aggregation behavior. The alimentary tract of insects, especially the hindgut, is colonized by opportunistic, facultative, and commensal microbiota defined by exogenous (diet and local environment) and endogenous (gut environment, hormones) factors (Douglas 2015). The commensal gut microbiota can modulate different aspects of insect biology, including host-parasite and host-pathogen interactions (Weiss 2006, Boissière et al. 2012), various life history traits (Ferrari and Vavre 2011, Douglas 2015), and behavior (Ezenwa et al. 2012, Wyatt 2014). Therefore, a plausible hypothesis is that in many insects, microbial fermentation components guide aggregation behavior, and multiple species have converged on overlapping microbial metabolites as aggregation cues.

Nevertheless, microbes can also produce unique blends of chemicals in members of the same species that can serve in individual recognition. The involvement of microbes in guiding individual recognition was summarized in the "fermentation hypothesis of chemical recognition" – bacteria within specialized scent glands produce essential mammalian scents that guide individual (within-species, e.g., hyenas) recognition (Albone and Perry 1976). Thus, variation in bacterial communities can drive variation in chemical recognition cues; use of learning and memory can make these reliable chemical signals for individual recognition. We sought to extend this idea to aggregations, and inquire whether fecal metabolites might serve not only to bring conspecifics together, but also to encode information about colony affiliation. That is, can the "fermentation hypothesis of [individual] chemical recognition" be extended to "fermentation hypothesis of [group] chemical recognition". Colony membership in social insects is usually guided by shared cuticular hydrocarbon (or other lipid) profiles as pheromonal nestmate recognition cues (van Zweden and d'Ettorre 2010). Recently, Vernier et al. (2020) demonstrated that shared gut microbiomes within colony members of honey bees produce nestmate recognition cues that contribute to inter-colony variation in semiochemicals, and in turn drive aggression on non-nestmates. Whether this observation can be extended to solitary insects that rest in aggregations remains unknown.

The German cockroach, *Blattella germanica* L., is a tractable experimental system in which to test these hypotheses. Aggregation behavior in this species is guided by a variety of cockroach-produced compounds (e.g., cuticular hydrocarbons and salivary compounds) (Ross and Tignor 1985, Rivault et al. 1998) and microbial metabolites produced by gut and fecal microbes (Wada-Katsumata et al. 2015). However, the chemical identities of aggregation pheromone components and their relative importance in guiding aggregation behavior have been contentious (Ishii and Kuwahara 1967, Ritter and Persoons 1974, McFarlane and Alli 1986, Sakuma and Fukami 1990, 1993, Rivault et al. 1998, Scherkenbeck et al. 1999, Wada-Katsumata et al. 2015, Hamilton et al. 2019). Disparate results may stem from methodological differences, such as extraction and purification of chemical fractions (e.g., Hamilton et al. 2019), different genetic stocks, or plasticity of the gut microbiota that might be shaped by diet and environmental conditions. The latter is a particularly appealing hypothesis – that the cockroach aggregation signal has broad phenotypic plasticity, and colonies fed different diets produce different aggregation signals, largely through changes in their gut microbiota. Indeed, Wada-Katsumata et al. (2015) suggested that the disparities in the bioactivity of two different carboxylic acid blends identified in two labs might derive from cockroaches discriminating between familiar and unfamiliar odor blends. Various interactions among members of an aggregation, such as coprophagy, further promote the within-group horizontal distribution and sharing of microbes. Thus, odors produced by the gut microbiota might allow cockroaches to recognize their own colony and distinguish it from other colonies.

Our overall goal was to test the plasticity of aggregation behavior to determine whether group recognition can lead to shelter fidelity in cockroaches. We accomplished this by manipulating the gut microbiota of cockroaches and therefore the microbially produced odors that guide aggregation behavior. Because the gut microbiome of *B. germanica* is strongly affected by diet composition (Perez-Cobas et al. 2015), we hypothesized that different diets would promote unique gut and fecal microbial assemblages that in turn would produce colony-specific aggregation odors. If cockroaches prefer familiar (learned) microbial odors over unfamiliar blends of odorants, they should prefer to aggregate in response to fecal odors from their own colony. We further hypothesized that cockroaches within aggregations obtain a core microbiota through coprophagy that contribute to the group odor. These inocula also mitigate low-quality diets, and we hypothesized that cockroaches that participate in coprophagy would experience faster development than cockroaches that did not consume feces. Our experimental design also examined whether cockroaches aggregate based on diet quality signaled by fecal metabolites (to fulfil nutritional needs) or in response to fecal odors that represent colony affiliation.

Materials and Methods

Cockroaches

An insecticide-susceptible strain of *B. germanica* (Orlando Normal = American Cyanamid, collected in a Florida apartment in 1947) was used in these experiments. Cockroaches were reared on rodent diet (Purina 5001 Rodent Diet, PMI Nutrition International, St. Louis, MO, USA) and were provided water in cotton-stoppered vials. Cockroach colonies were maintained at 27 ± 1 °C, 40–70% relative humidity, and L:D = 12:12 h photoperiod.

Sterilization of Egg Cases

B. germanica embryos that are close to hatching develop a prominent green spot, representing yolk in the midgut, and pigmented eyes. Gravid females with egg cases showing a "green line" were anesthetized with CO_2 and the egg cases were gently removed with forceps. All solutions were made with autoclaved water. Egg cases were sterilized by first washing them for 1 min with a 0.5% bleach solution in water, then 70% ethanol in water for 1 min, and three times with water for 1 min each. To determine if the sterilization process was effective, two surface-sterilized egg cases were plated on tryptic soy agar (TSA) and plate count agar (PCA). Egg cases were tested for culturable microbes both on their surface and in crushed samples. After three days of incubation at 28°C, no visible colonies were observed in any of the treatments. Additionally, an aliquot of the last washing step was plated on three TSA plates and after three days of incubation at 28°C, a single colony was observed on only one of the plates. The control plates were clean, and no contamination was detected.

To generate the three experimental groups with three different types of diets, 20–30 sterilized egg cases were placed in 15 mL conical centrifuge tubes (Falcon-Corning, Corning, NY, USA) and kept in a 18.7 x 13.3 x 9.5 cm rearing cage (T-79, Althor Products, Windsor Locks, CT, USA) that had been sterilized with bleach and 95% ethanol. To maintain moisture, glass test tubes containing either distilled water or autoclaved water were placed in the cage until the eggs hatched. After hatching, first instar nymphs were divided into three sterile rearing cages (cleaned with 90% ethanol in water solution).

Three Types of Diets

To obtain cockroach feces of different qualities for the aggregation bioassays, three diets were chosen based on their different protein, carbohydrate, lipid, and fiber content: cat diet (Taste of the Wild Rocky Mountain Feline Recipe with roasted venison and smoked salmon, Schell & Kampeter Inc., Meta, MO, USA) was a high protein, low carbohydrate and low fiber diet (42% protein, 21% carbohydrate, 18% lipid, 3% fiber); rabbit diet (Oxbow Garden Select Adult Rabbit Food, Oxbow Enterprises Inc., Murdock, NE, USA) was a low protein, high carbohydrate and high fiber diet (12% protein, 43% carbohydrate, 2.5% lipid, 22–26% fiber); rodent diet was the same diet used in regular rearing – moderate protein, high carbohydrate, low fiber (24% protein, 58% carbohydrate, 5% lipid, 5.2% fiber). Each diet was crushed in a blender and placed into separate Ziploc bags. Sterilized rodent diet was also prepared using gamma radiation at a dose of 10,000 Gy at the USDA-APHIS-PPQ S&T Otis Laboratory (Buzzards Bay, MA). To verify the sterilization process, two samples of the irradiated diet were plated on TSA and PCA media using 4 plates per sample. After 3 days of incubation at 28°C, we detected no colonies or visible growth in any of the diet and control plates.

Experimental Groups

Three experimental groups were generated, all using nymphs that hatched from sterilized egg cases: Diet-only, Feces (from rodent diet)-and-Diet, and Feces (from various diets)-and-Sterilized rodent diet. Within each group, nymphs either fed on different diets or the feces of cockroaches fed different diets. Cockroach colonies were maintained at 27 ± 1°C, 15–30% relative humidity, and L:D = 12:12 h photoperiod. It is important to reiterate that the egg case sterilization procedure resulted in neonates that lacked a gut microbiome, but they retained their maternally provisioned endosymbionts, namely *Blattabacterium*.

<u>Diet-only Group</u>. The purpose of this group was to provide sterile cockroaches with different non-sterile diets that would be expected to inoculate them and support different gut microbiota. These colonies would then produce feces with different microbial communities. We expected that the fecal microbiome on all three diets would contain some core microbes that would be shared across all three colonies, but also that each diet would support some colony/diet-specific microbes. First instar nymphs that hatched from sterilized egg cases were divided into three rearing cages that were first sterilized with ethanol and contained egg carton shelters, autoclaved water in a glass vial, and diet (~1 g of cat, rabbit, or rodent diet) placed in a disposable plastic Petri dish (5.5 cm diameter, Falcon-Corning). There were two replicate cages per diet. The cockroaches were allowed to develop and the dates that each instar stage was

reached were recorded for each cage. The cockroaches were moved to clean rearing cages every 2–3 weeks, but the same shelters were transferred with them. Water and diets were replaced as needed. The dates of adult emergence in each cage were recorded, as were any mating events we observed. When adult females developed egg cases, they were moved to a clean rearing cage with shelter, water, and the same diet. Because gravid German cockroach females feed very little, we removed the egg cases from half of the gravid females, and placed the females in a clean rearing cage with shelter, water, the same diet, and a screen mesh bottom to collect feces for behavioral assays. This procedure accelerates the onset of a new gonotrophic cycle and feeding. When egg cases hatched, the feces was collected from each cage and first instar nymphs were used in behavioral assays.

<u>Feces (from rodent diet)-and-Diet Group</u>. The purpose of this group was to first inoculate cockroaches with a microbe-rich common fecal source and then provide them different diets that would support colony-specific microbial communities in their gut and feces. These microbial communities were expected to have some shared microbes and some colony-specific microbes. This group also served to investigate the effects of coprophagy on cockroach development. First instar nymphs that hatched from sterilized egg cases were divided into three rearing cages that had been sterilized with ethanol and contained egg carton shelters, water in a glass vial, and ~0.5 g feces collected from females in the laboratory colony (rodent diet fed) and placed in a disposable plastic Petri dish. Nymphs were allowed to feed on feces only for three days and then the feces was removed. One non-sterile diet (~1 g cat, rabbit, or rodent diet) in a plastic Petri dish was then added to each cage. There were two replicate cages

per diet. Cockroach development was recorded for each cage using the same method as with the Diet-only group. The cockroaches were moved to clean rearing cages every 2–3 weeks, the same shelters were kept, and water and diets were replaced as needed. Gravid females were moved to a clean rearing cage with shelter, water, and the same diet. As before, the egg cases were removed from some females and they were placed in a clean rearing cage with shelter, water, the same diet, and a screen mesh bottom to collect feces for behavioral assays. First instar nymphs that hatched in this group were used in behavioral assays and feces was collected from each cage for extraction.

<u>Feces (from various diets)-and-Sterilized rodent diet Group</u>. The purpose of this group was to inoculate sterile first instar nymphs with colony-specific microbes by providing them feces produced by cockroaches fed different non-sterile diets. These cockroaches were then given the same sterilized rodent diet in a sterile environment that would be expected to support colony-specific gut microbiota representing subsets of the shared inoculum. All materials used for this group were autoclaved and all handling and maintenance of cockroaches were done in a biosafety cabinet. First instar nymphs that hatched from sterilized egg cases were divided into three rearing cages. Aeration of the cages was through nylon membrane filters (Sterlitech, 0.2 µm mesh, 200 x 200 mm, Kent, WA) that were first cleaned with ethanol and sterilized under UV light overnight. After placing an autoclaved egg carton shelter and an autoclaved water vial in the rearing cage, we provided the nymphs ~0.5 g non-sterile feces from females in the Feces (from rodent diet)-and-Diet group in a disposable plastic Petri dish. We expected that the three types of feces produced from cockroaches fed different diets would contain different profiles of

fecal microbes. To inoculate them with fecal microbiota, nymphs were allowed to feed on feces-only for three days. The feces was replaced with sterilized rodent diet (~1 g) in a plastic Petri dish. There were two replicate cages per feces type, and both cages were placed in a large 45.7 x 30.5 x 22.9 cm plastic container (5-gal Clear Tote Box, Rubbermaid Commercial Products, USA) cleaned with 90% ethanol in water solution and sterilized under UV light overnight. Cockroach development was observed and recorded for each cage weekly. Sterilized water and diets were replaced as needed. Adult females and gravid females were separated into clean rearing cages with shelter, water, and sterilized rodent diet. Feces was collected from each cage and newly hatched first instar nymphs were used in behavioral assays. To assess the importance of fecal inoculation of first instar nymphs, we also set up a rearing cage with sterile first instar nymphs that were provided only sterilized rodent diet and no feces under the same environmental conditions, and cockroach development in this cage was also recorded.

Metrics of Cockroach Development

In addition to recording the first day nymphs reached each instar stage and the emergence days of adults in each cage, we also monitored the number of days from the first to last adult emergence for each group. Mortality and weight of adult males and females (0–1 days old) was recorded in the Diet-only group and the Feces (from Rodent diet)-and-Diet group.

Sampling of Cockroaches and Feces for Microbiome Analysis

For microbiome analysis (to be reported separately), at least 10 adult females and 10 males were collected from the Diet-only group, Feces (from Rodent diet)-and-Diet group, and Feces (from various diets)-and-Sterilized rodent diet group. Adults were stored individually in 1.5 mL Eppendorf tubes in a -20°C freezer, along with fecal samples of females from the three groups.

Bioassays Using First Instar Nymphs

Preparation of Fecal Extracts. Fecal extracts were prepared by adding 25 mg of adult female feces to 0.5 mL of either distilled water or autoclaved water in 1.5 mL Eppendorf tube. The suspension was vortexed well for 3 min, then centrifuged (14,000 rpm for 1 min at 4°C). The supernatant was collected in a 1.5 mL Eppendorf tube. We defined this initial extract as Concentration "1" (50 µg feces-equivalents/µL). To determine the concentration of this fecal extract to use in two-choice aggregation assays, the initial extract was diluted to 0.1 (5 µg feces-equivalents/µL) and 0.01 (0.5 µg feces-equivalents/µL) with either distilled water or autoclaved water for dose-response assays.

Dose-Response Assays. Using individual first instar nymphs (2–3 days old) from each group, two-choice aggregation assays were conducted in plastic Petri dishes (5.5 cm diameter) during the photophase. Two accordion-style folded filter paper shelters (2.0 x 0.7 cm, Whatman #1, Pittsburgh, PA) were placed at opposite edges of the arena. One shelter was treated with 1 µL of fecal extract obtained from adult females of their own group. The other shelter was treated as a control with 1 µL of water. To evaluate any directional bias in the assay conditions, nymphs were given a choice between two water-treated shelters. A single first instar nymph was introduced in the center of the arena, and the position of the nymph within the arena was recorded 2 hrs later. We conducted 20-60 assays per concentration. To standardize the stimuli in each subsequent two-choice aggregation assay, we needed to estimate the 50% Effective Concentration (EC_{50}) of each fecal extract. The response in the dose-response curve was calculated as follows: % choosing the fecal extract = 100*[(# of nymphs choosing fecal extract - # of nymphs choosing water) / total # of responders]. The EC_{50} was estimated from the resulting dose-response curve for each group, and used in the aggregation assays.

Aggregation Assays. Two-choice aggregation assays were performed using individual first instar nymphs in plastic Petri dishes (9 cm diameter) during the photophase. First instar nymphs were given a choice between two folded tent-shaped filter papers (2 x 2 cm). One filter paper shelter was treated with 3 µL of fecal extract from their own group, and the other filter paper shelter was treated with 3 µL of fecal extract from one of the other groups. The various treatments for all the groups were (1) own group fecal extract vs. water; (2) fecal extracts from nymphs fed rodent vs. rabbit diets; (3) fecal extracts from nymphs fed rodent vs. cat diets; and (4) fecal extracts from nymphs fed rabbit vs. cat diets. The position of each nymph was recorded 2 hrs later and there were 40–80 assays per treatment.

Statistics

Unpaired *t*-tests were conducted to compare development of adult males and females on each diet in the Diet-only group and the Feces (from rodent diet)-and-Diet group. One-way analysis of variance (ANOVA) was used to compare the body mass of adults in each diet treatments for the Diet-only group and the Feces (from rodent diet)-and-Diet group, and to compare the diet treatments within the Diet-only group and the Feces (from rodent diet)-and-Diet group separately. Each ANOVA was followed by Tukey-Kramer's honestly significant difference (HSD) test ($\alpha = 0.05$) to identify differences among experimental treatments. Differences in responses to the two filter paper shelters in two-choice bioassays were assessed using a Chi-square test of independence ($\alpha = 0.05$) for all experimental groups. Dose-response curves for each group and all statistical analyses were performed in R 4.0.2 software.

Results

Effects of Diet and Feces Under Non-Sterile Conditions

Development. Nymphal development rates on the three different diets in both the Diet-only group and the Feces (from rodent diet)-and-Diet group are shown in Fig. 1. For the Diet-only group, nymphs that fed on rodent diet took 36.5 days to reach the adult stage, nymphs that fed on cat diet took 36 days, and nymphs that fed on rabbit diet took 39 days to become adults (Fig. 1A). The addition of feces in the Feces (from rodent diet)-and-Diet group did not accelerate development – nymphs that fed on rodent diet took 37.5 days, nymphs that fed on cat diet took 37 days, and nymphs that fed on rabbit diet became adults after 41 days (Fig. 1B). Nymphal development rates were similar in the two groups even though nymphs in the Feces (from rodent diet)-and-Diet group received feces during the first three days of the first instar stage. It is important to note that nymphs in the Feces (from rodent diet)-and-Diet group experienced a slight delay in development because they were deprived of food for the first 3 days while provided feces only. For both groups, nymphs that fed on rabbit diet had longer development periods than nymphs fed either rodent or cat diets. These results indicate that the low protein, high carbohydrate and high fiber rabbit diet was a suboptimal diet, and that the addition of feces (and associated microbiota) did not overcome the effects of low diet quality.

Similar results were found for the times for all nymphs to become adults (first to last emergence day). For the Diet-only group, it took 9.5 days for all nymphs that fed on rabbit and cat diet to become adults, whereas cockroaches that fed on rabbit diet took 17 days (Fig. 1C). For the Feces (from rodent diet)-and-Diet group, it took 12.5 days for all nymphs that fed on rodent diet to become adults, 10 days for nymphs that fed on cat diet, and 14 days for all nymphs that fed on rabbit diet to become adults (Fig. 1D). For both groups, it took longer for all nymphs that fed on rabbit diet to reach the adult stage than nymphs that fed on either rodent or cat diet, which again indicates that the rabbit diet was of lesser quality for cockroaches than the other two diets.

The Diet-only group fed rodent diet experienced low (9%) mortality through nymphal development, whereas cockroaches fed cat or rabbit diet had double the mortality (20% and 21%, respectively). Surprisingly, the addition of feces in the Feces (from rodent diet)-and-Diet group resulted in greater mortality on all three diets – 26%, 31% and 45.5% on rodent, cat and rabbit diets, respectively. Thus, as with nymphal development and adult emergence, mortality throughout development indicated that rabbit diet was inferior, and the addition of cockroach feces did not provide additional benefits to the cockroaches.

Generally, adult body mass followed a similar pattern as other metrics of development. First, adult females attained a higher body mass than males in each of the six diet treatments (three diets in the Diet-only group and three diets in the Feces (from rodent diet)-and-Diet group) (Table 1A). Second, there were no significant differences in adult body mass of cockroaches that fed on the same diet in the two treatment groups (Fig. 2, Table 1B). And third, within each treatment group, there were significant differences in adult body mass of cockroaches fed different diets; in both the Diet-only group and the Feces (from rodent diet)-and-Diet group, cockroaches fed rabbit diet attained lower body mass than those fed cat or rabbit diet (Fig. 2, Table 1B). As with other developmental parameters, these results show that feeding on feces did not make a significant difference in adult body mass, whereas diet quality significantly affected adult body mass. Overall, nymphs that fed on rabbit diet had longer development times, greater mortality, and lower adult body mass, indicating that rabbit diet is nutritionally suboptimal for cockroaches. The addition of cockroach feces during the first instar stage, when neonates engage in coprophagy, did not enhance cockroach development, the period of adult emergence, or adult body mass, and did not reduce mortality. Thus, diet quality appears to be critical for cockroach development, and any nutrients and microbes obtained from conspecific feces could not overcome the negative effects of low-quality diet.

Dose-Response Aggregation Assays. We conducted dose-response sheltering assays to estimate appropriate doses that could be used in two-choice bioassays of fecal extracts. The dose-response curves and estimated EC_{50} for the Diet-only group and the Feces (from rodent diet)-and-Diet group are shown in Fig. 3. We found no side bias in any of the two-choice assays, as nymphs equally chose the two water-treated shelters. When given a choice between a filter paper shelter treated with water and a shelter treated with fecal aqueous extracts, nymphs preferred the fecal extracts in a dose-dependent manner. The dose-response curves were similar for all six treatments (three diets x two treatment groups). Therefore, in all subsequent two-choice aggregation assays, we used the 0.1 concentration of fecal extract (water extract of 5 µg feces applied to filter paper in 1 µL), representing approximately the EC_{50}.

Aggregation Assays. Results from the two-choice aggregation assays for the Diet-only group and the Feces (from rodent diet)-and-Diet group are shown in Fig. 4. There was a high number of responders (>85%) for all aggregation assays when the 0.1 concentration of fecal extract was used. For both groups, nymphs significantly preferred to rest under filter paper shelters treated with fecal extracts over filter paper shelters treated with water only (Fig. 4A, B, Table 2). When nymphs were given a choice between two filter paper shelters treated with different fecal extracts, nymphs from both the Diet-only and the Feces (from rodent diet)-and-Diet groups preferred their own colony's fecal extracts. For the Diet-only group, nymphs that fed on rodent diet significantly preferred the fecal extracts from colonies fed rodent diet over cat or rabbit diet, nymphs fed a cat diet preferred fecal extracts from cat diet-fed colonies over rodent or rabbit diet, and nymphs that fed on rabbit diet preferred to shelter on fecal extracts from rabbit diet-fed colonies over rodent or cat diets (Fig. 4A, Table 2). We found similar results for the Feces (from rodent diet)-and-Diet group, where in all cases, nymphs consistently preferred to shelter on fecal extracts produced by the same diet that they were fed (Fig. 4B, Table 2). These results suggest that diet is important in colony recognition and that cockroaches can discriminate their own fecal odors as aggregation signals from foreign cockroach fecal odors. When nymphs from either the Diet-only group or the Feces (from rodent diet)-and-Diet group were given a choice of filter paper shelters treated with their own colony's fecal extract or with fecal extracts from the other experimental group that fed on the same diet, there was no significant preference for either fecal extract (Fig. 4C). Nymphs from both groups did not discriminate their own colony's fecal extracts from the fecal extracts of cockroaches that fed on the same diet, suggesting that diet quality shaped the microbial communities in cockroach feces and the metabolites they produced.

Effects of Diet and Feces Under Sterile Conditions

Development. All neonates that fed on non-sterile feces produced by colonies fed rodent, cat, or rabbit diets and then provided sterile rodent diet had similar development times, reaching the adult stage in 36–37 days (Fig. 5A). These results show that although they received feces from different quality diets, feeding on the same sterile rodent diet resulted in similar nymphal development rates. The times for all nymphs to become adults (first to last emergence day), varied with the quality of feces the nymphs ate. It took 18 days for all nymphs that fed on cockroach feces derived from rodent diet-fed cockroaches to become adults, 14 days for nymphs that fed on feces from cockroaches that were fed cat diet, and 13 days for all nymphs that fed on cockroach feces from females fed rabbit diet to become adults (Fig. 5B). There were no observable differences in development or adult emergence times for nymphs in this group that fed only on sterilized rodent diet. We expected nymphs in this group to have similar adult emergence times to nymphs in the Feces (from rodent diet)-and-Diet group whose feces they received. However, nymphs fed cockroach feces from females that fed on rabbit diet took less time to reach the adult stage than nymphs fed rabbit diet in the Feces (from rodent diet)-and-Diet group. Nymphs fed feces from females that were fed rodent diet took more time to reach the adult stage than nymphs fed rodent diet in the Feces (from rodent diet)-and-Diet group

(Fig. 1B). These results suggest that development could be improved when nymphs were offered a higher quality diet, at least in the case of nymphs in the Feces (from various diets)-and-Sterilized rodent diet group that fed on feces from cockroaches fed rabbit diet. While these nymphs were fed feces from females that fed on a low-quality diet, feeding on sterilized rodent diet improved their development time and shortened the time for all nymphs to reach the adult stage. These results suggest that nutrients in the diet are more important for cockroach development than microbes obtained from fecal materials through coprophagy.

Dose-response Aggregation Assays. The dose-response curves and estimated EC_{50} for this group (Fig. 5C) were similar to the patterns we obtained with the Diet-only group and the Feces (from rodent diet)-and-Diet group (Fig. 3). As before, we detected no side bias in any of the dose-response assays, and nymphs preferred the fecal extracts over the water control in a dose-dependent manner. Again, the 0.1 concentration of fecal extract (water extract of 5 µg feces applied to filter paper in 1 µL), representing approximately the EC_{50}, was used in all subsequent two-choice aggregation assays.

Aggregation Assays. Non-sterile feces from cockroaches fed rodent, cat or rabbit diets was used to inoculate sterile nymphs through coprophagy, and these nymphs and the adults that followed were fed sterile rodent diet. The neonates that subsequently hatched were used in aggregation bioassays. A high percentage of these nymphs responded (>85%) in all assays, and they significantly preferred to rest under filter paper shelters treated with fecal extracts over filter paper shelters treated with water (Fig. 5D, Table 2). However, unlike the previous groups that we tested, these nymphs did not show a preference in two-choice assays when both choices were treated with fecal extracts (Fig. 5D, Table 2). That is, although the feces generated from different diets was expected to inoculate the nymphs with different microbial communities, eating the same diet throughout nymphal development and during the first gonotrophic cycle likely converged the fecal microbiomes and thus prevented nymphs in bioassays from discriminating their own from other colonies' fecal odors.

Discussion

Manipulation of the Cockroach Fecal Microbiota

Our goal was to investigate the impact of different diets and feces on development and aggregation behavior of *B. germanica* under both non-sterile and sterile conditions. A major strategy in these experiments was to manipulate the gut microbial communities of cockroaches by raising them on diets of varying nutritional quality, and by providing them feces from cockroaches fed different diets. Six lines of reasoning support the rationale of this strategy. First, *B. germanica*, like other cockroach species, hatch devoid of a gut microbiome, and they inoculate their gut mainly through coprophagy (Carrasco et al. 2014, Rosas et al. 2018). This supports our approach of sterilizing the egg case to obtain first instars with axenic guts. Second, aggregation behavior in *B. germanica* was found to be stimulated by volatile carboxylic acids in feces extracts that were absent when axenic nymphs were produced by surface-sterilizing the egg cases (Wada-Katsumata et al. 2015). Therefore, using cockroaches hatched from surface-sterilized egg cases allowed us to experiment with cockroaches that were devoid of gut microbes and contained only vertically-transmitted *Blattabacterium*. Third, Kakumanu et al.

(2018) demonstrated that 80–90% of the bacterial operational taxonomic units were shared between the feces and the respective gut microbiota in both lab-reared and field-collected *B. germanica*, showing that the fecal microbiota is a fair representative of the gut microbiome. Notably, the microbiome of adult females and males were similar within a location, justifying the use of female feces in our experiments. Fourth, in support of the broad overlap between the gut and fecal microbiomes, the presence of feces from healthy cockroaches could overcome disruptions of the gut microbiome of *B. germanica*, such as from antibiotics (Dominguez-Santos et al. 2020). Fifth, the gut microbiome of omnivorous *B. germanica* is highly dynamic, as it responds rapidly to different diets and reassembles in a diet-specific manner; low- or no-protein diets support higher bacterial diversity than high-protein diets (Perez-Cobas et al. 2015). Greater variation in the microbial community composition of feral cockroaches collected in apartments than in lab-reared cockroaches (Kakumanu et al. 2018) also supports the idea that food and the local environment are major factors that shape the gut and fecal microbiome. Finally, cockroaches from various field sites shared thousands of core bacterial taxa (Kakumanu et al. 2018). These features of the *B. germanica* system provide rationale for our hypothesis that different diets would maintain divergent fecal microbiomes across cockroach populations, which in turn would generates a large array of microbial metabolites, with some overlap among the populations. Such phenotypic plasticity in aggregation signals could lay the foundation for population-specific and aggregation-specific signals and 'colony'-recognition. These features of *B. germanica* also supported our three-pronged approach of starting with axenic nymphs, inoculating them with microbes from cockroach feces, and altering their gut microbiota with diets of different qualities. By providing cockroaches different diets (Diet-only group), we were able to obtain cockroach feces that was expected to vary in its microbiota. Thus, differences in aggregation responses among first instar nymphs can be attributed to the microbial metabolites in the fecal extracts.

Role of Fecal Metabolites in Colony Recognition

There are two major categories of aggregation cues. The first would be species-specific pheromones that elicit species-specific behaviors and physiological responses. These signals are often single compounds or defined blends of compounds that are integrated with visual and tactile cues to stimulate aggregation. For example, aggregation pheromone in desert migratory locust (*Locusta migratoria*) drives solitary adults that encounter pheromone-emitting groups of gregarious locusts to become arrested and recruited into the groups; contact with gregarious locusts then causes the solitary locusts to acquire gregarious characteristics, including changes in color and morphology (review: Ahmed et al. 2005). Gut bacteria appear to be involved in the production of the locust aggregation pheromone, and also contributed to locust defense against microbial insect pathogens by producing antimicrobial compounds (Dillon et al. 2002). Recently, Guo et al. (2020) identified the aggregation pheromone as 4-vinylanisole. Although other compounds might contribute to a blend that elicits aggregation behavior, 4-vinylanisole nonetheless represents an invariable, species-specific aggregation signal that acts as species-specific sex pheromones do. It remains unknown, however, whether 4-vinylanisole is produced by gut or fecal microbes, or both. This system represents an ecological setting where species recognition is important, but group membership (e.g., resting aggregation, kin) is likely not encoded in the pheromonal signal.

A second class of aggregation pheromones consists of phenotypically plastic blends that are subject to variation based on diet and other environmental factors. These pheromones are used to discriminate between members and non-members of an aggregation or colony. In eusocial insects, cuticular hydrocarbons generally serve as colony recognition signals that prevent non-colony members from exploiting the colony's resources (van Zweden and d'Ettorre 2010). In gregarious cockroaches, such as *B. germanica*, various metabolites in feces, mainly volatile carboxylic acids, serve as aggregation signals (Wada-Katsumata et al. 2015). Both of these systems (cuticular hydrocarbons and fatty acids) are subject to changes by diet and contact with other insects, and interestingly, recent evidence suggests some convergence between these two seemingly disparate sources. For example, microbial symbionts are also involved in the production of aggregation pheromones and compounds involved in nestmate recognition (Engl and Kaltenpoth 2018). In the termite *Reticulitermes speratus* and the harvester ant *Pogonomyrmex barbatus*, bacterial associates have an effect on the hosts' chemical profiles and therefore on recognition (Matsuura 2001, Dosmann et al. 2016). Thus, in social insects, shared food, environmental materials, and gut microbiomes within a colony contribute to unique blends of cuticular hydrocarbons and other nestmate recognition signals that differentiate the colony from other colonies (Leonhardt et al. 2016, Vernier et al. 2020).

German cockroaches can discriminate between strain odors (Rivault and Cloarec 1998). In mixed aggregations, chemical exchanges between individuals may accelerate the appearance of a new chemical signature, which reinforces the mixing of the strains (Ame et al. 2004). Although the underlying mechanism may be similar to numerous observations in ant colonies (e.g., Dahbi et al. 1997, Dahbi and Lenoir 1998) that involve cuticular hydrocarbons, in cockroaches it most likely involves the sharing of fecal microbes via coprophagy.

Our findings support the idea that the German cockroach uses the second category of aggregation pheromones – highly plastic microbial metabolites that can vary based on diet and possibly other environmental conditions. Our findings support the idea, first suggested by Wada-Katsumata et al. (2015), that cockroaches use odors produced by colony-specific microbes to recognize and aggregate with colony members. Overall, we found that first instar nymphs significantly preferred to aggregate with fecal extracts produced by adult females from their own colony over fecal extracts produced by a different colony that fed on a different diet. The same trend in aggregation preferences was found in the Feces (from rodent diet)-and-Diet group. These cockroaches were first inoculated with microbes from feces generated by cockroaches that fed on rodent diet, split into three treatment groups, and provided three different non-sterile diets. When nymphs from both experimental groups were given a choice between their own colony fecal extract and the fecal extracts from the other experimental group that fed on the same diet, there was no significant preference for either extract, indicating that diet had a strong impact on microbial communities in the feces. These results provide further evidence that diet has a substantial influence on aggregation preference and colony recognition. Notably, we also concluded that aggregation behavior was not based on fulfilling nutritional needs, because cockroaches raised on the low-quality rabbit diet still preferred their own fecal extract (presumably of low nutritional quality) over the higher quality fecal extracts of cockroaches that fed on higher-quality rodent or cat diets.

Role of Coprophagy and Diets in Cockroach Development

Microbes offer a range of benefits to insects that include contributions to nutrition, digestion, and defense, and participate in the production of chemicals that mediate communication between conspecifics. While endosymbionts are vertically maternally transmitted, the transmission of beneficial gut symbionts to the next generation primarily occurs horizontally through social contact such as coprophagy in gregarious insects and trophallaxis in eusocial insects (Onchuru et al. 2018). Coprophagy serves two major functions in *B. germanica* – it inoculates neonate cockroaches with fecal microbes that shape the gut microbial community, and it provides significant nutritional benefits to first instars. In the American cockroach (*Periplaneta americana*), gut bacteria inoculated through coprophagy are essential for normal gut tissue and organismal development (Jahnes et al. 2019).

In cockroaches, nymphs hatch with an alimentary tract devoid of microbes. The gut microbiota is then horizontally transferred via coprophagy (Carrasco et al. 2014). Thus, the first instar is inoculated and a large increase in bacterial quantity and diversity occurs after the first molt, after which bacterial load and richness remain relatively constant (Carrasco et al. 2014). Different studies show conflicting roles of the gut microbiome in cockroach nutrition. This is likely related to the experimental design – the effects of gut microbes may be evident only under stressful conditions, such as nutrient-deficient or imbalanced diets. Our study was not designed to assess the nutritional benefits of the gut microbiota. Rather, we used nutritionally divergent diets to establish divergent gut microbiomes, but all three diets were relatively nutritious. Future studies might compare performance of cockroaches with or without a gut microbiota when provided a nutritionally imbalanced diet, such as rabbit diet.

Coprophagy in cockroaches could enhance nutrient acquisition without requiring nymphs to forage and thus serve as an important nutritional buffer for first instar nymphs. The first instar stage is far less likely than older nymphs to forage long distances from a shelter and are more likely to engage in coprophagy to obtain nutrients (Cloarec and Rivault 1991, Kopanic Jr and Schal 1999). When provided no source of food other than conspecific feces, neonate *B. germanica* survived longer than first instar nymphs deprived of feces; some molted to the second instar, which is much more mobile and relatively less dependent on coprophagy (Kopanic Jr et al. 2001). Moreover, the nutritional quality of adult feces was found to be influenced by the adult diets (Kopanic Jr et al. 2001). Again, the aim of our study was to manipulate the fecal microbiota, and it was not powered to assess the nutrient value of feces. Indeed, it was apparent from our results that nutrients obtained from the diet had a much greater impact on nymphal development than any nutrients potentially obtained from adult female feces. The addition of feces in the Feces (from rodent diet)-and-Diet group did not accelerate development of nymphs compared to cockroaches in the Diet-only group and it did not mitigate the negative effects of the lower-quality rabbit diet.

Nymphal development is affected significantly by both the quality and quantity of protein in their diet (Cooper and Schal 1992). This was also reflected in our results where cockroaches that fed on the low-protein high-fiber rabbit diet had longer nymphal development times and longer adult emergence times than cockroaches that fed on either the cat diet (high protein, low fiber) or rodent diet (moderate protein, low fiber).

In summary, *B. germanica* aggregations promote growth and survival of nymphs, increase foraging efficiency, support mate location, and may contribute to thermoregulation and mitigate desiccation. Many factors affect aggregation behavior in the German cockroach, including shelter size, texture and orientation, presence of conspecifics, pheromones, and other factors (reviews: Rust et al. 1995). Cockroach aggregation stimuli vary in their quality and quantity and are dependent to a large extent on environmental factors such as food quality, ingested microbes, and possibly the genetic background of cockroach populations (Wada-Katsumata et al. 2015). The results of this study demonstrated that diet affected the quality of fecal extracts and in turn, the aggregation preferences of first instar nymphs. It is highly likely that the diets altered the gut microbial communities responsible for producing aggregation pheromones. A clearer causative relationship will need to await results from the follow-up microbiome and chemical analyses to establish the association between behavioral preferences, the respective microbial communities in the gut and feces, and the semiochemicals emitted from feces.

Acknowledgments

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Table 1. Statistical comparisons between adult male and female body mass in each diet treatment for Diet-only and Feces (from rodent diet)-and-Diet groups (A) and among all diet treatments in both Diet-only and Feces (from rodent diet)-and-Diet groups for adult males and females (B).

A.

Differences between the body mass of males and females in each experimental group	
	***t*-test**
Rodent Diet-only	$t = 27.40$, $p < 0.0001$
Feces (from rodent diet)-and-Rodent diet	$t = 24.43$, $p < 0.0001$
Cat Diet-only	$t = 25.05$, $p < 0.0001$
Feces (from rodent diet)-and-Cat diet	$t = 26.84$, $p < 0.0001$
Rabbit Diet-only	$t = 23.44$, $p < 0.0001$
Feces (from rodent diet)-and-Rabbit diet	$t = 18.27$, $p < 0.0001$

B.

Differences between all experimental groups		
	Males	**Females**
ANOVA	$F\ (5, 447) = 14.81$ $p < 0.001$	$F\ (5, 436) = 11.07$ $p < 0.001$

Table 2. Statistical comparisons of aggregation preference of first instar nymphs for filter paper shelters treated with either their own colony fecal extract or a different colony fecal extract.

Experimental Group	Aggregation Test	*n*	Chi-square test
Rodent Diet-only	**Rodent vs Water**	40	χ2(1) = 17.86 $p < 0.0001$
	Rodent vs Cat	40	χ2(1) = 5.765 $p = 0.0164$
	Rodent vs Rabbit	40	χ2(1) = 4.829 $p < 0.0001$
Cat Diet-only	**Cat vs Water**	50	χ2(1) = 14.08 $p = 0.0002$
	Cat vs Rodent	60	χ2(1) = 4.571 $p = 0.0325$
	Cat vs Rabbit	60	χ2(1) = 8.345 $p = 0.0039$
Rabbit Diet-only	**Rabbit vs Water**	40	χ2(1) = 15.11 $p = 0.0001$
	Rabbit vs Rodent	40	χ2(1) = 5.444 $p = 0.0196$
	Rabbit vs Cat	40	χ2(1) = 14.24 $p = 0.0001$
Feces (from rodent diet)-and-Rodent diet	**Rodent vs Water**	70	χ2(1) = 33.99 $p < 0.0001$
	Rodent vs Cat	60	χ2(1) = 35.53 $p < 0.0001$
	Rodent vs Rabbit	56	χ2(1) = 27.94 $p < 0.0001$
Feces (from rodent diet)-and-Cat diet	**Cat vs Water**	79	χ2(1) = 37.39 $p < 0.0001$
	Cat vs Rodent	80	χ2(1) = 18.51 $p < 0.0001$
	Cat vs Rabbit	76	χ2(1) = 14.92 $p = 0.0001$
Feces (from rodent diet)-and-Rabbit diet	**Rabbit vs Water**	62	χ2(1) = 32.27 $p < 0.0001$
	Rabbit vs Rodent	80	χ2(1) = 12.16 $p = 0.0004$
	Rabbit vs Cat	80	χ2(1) = 23.84 $p < 0.0001$

Table 2 (continued).

Feces (from rodent diet)-and-Sterilized rodent diet	**Rodent vs Water**	60	$\chi2(1) = 27.66$ $p < 0.0001$
	Rodent vs Cat	60	$\chi2(1) = 0.0169$ $p = 0.8964$
	Rodent vs Rabbit	60	$\chi2(1) = 0.0667$ $p = 0.7963$
Feces (from cat diet)-and-Sterilized rodent diet	**Cat vs Water**	75	$\chi2(1) = 32.01$ $p < 0.0001$
	Cat vs Rodent	75	$\chi2(1) = 0.0541$ $p = 0.8162$
	Cat vs Rabbit	60	$\chi2(1) = 0.2857$ $p = 0.5930$
Feces (from rabbit diet)-and-Sterilized rodent diet	**Rabbit vs Water**	75	$\chi2(1) = 31.11$ $p < 0.0001$
	Rabbit vs Rodent	75	$\chi2(1) = 0.2222$ $p = 0.6374$
	Rabbit vs Cat	75	$\chi2(1) = 2.8$ $p = 0.0943$

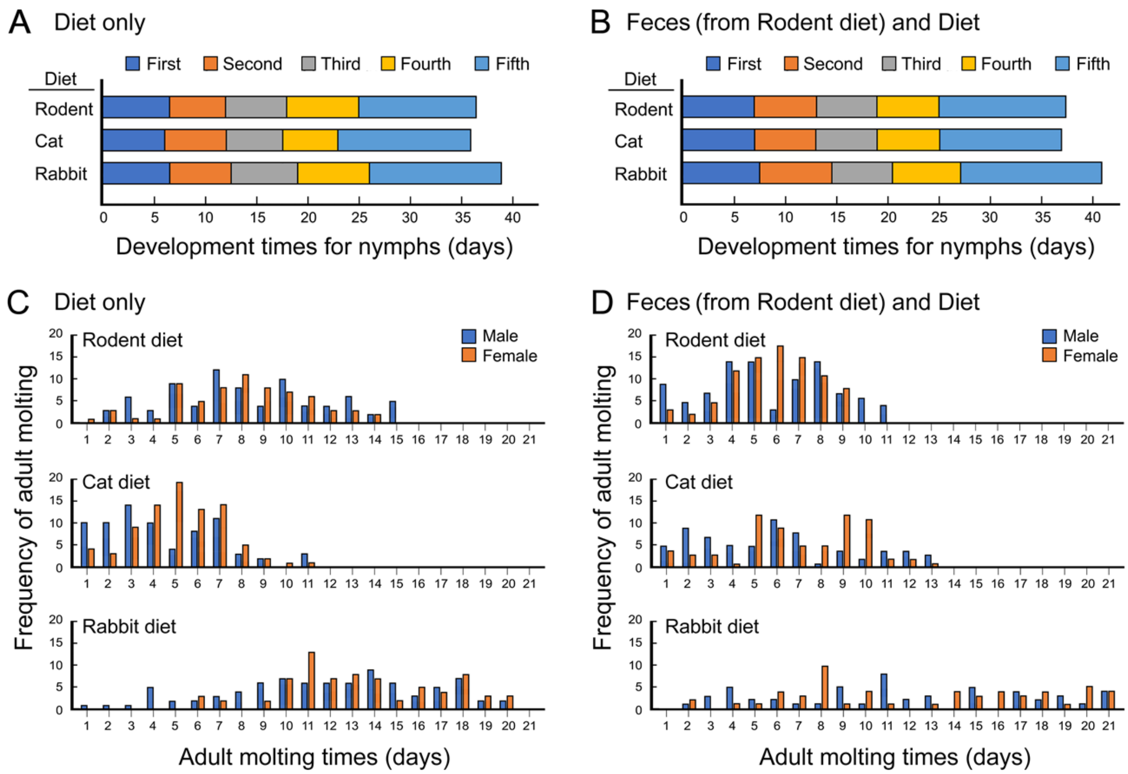


Figure 1. Development periods for Diet-only and Feces (from Rodent diet)-and-Diet groups. Average number of days nymphs fed different diets in the (A) Diet-only and (B) Feces (from Rodent diet)-and-Diet groups spent in each instar stadium. Different colored bars represent different nymphal stages. Average number of males and females fed different diets molting on each day from the first to last days of adult emergence in the Diet-only (C) and Feces (from Rodent diet)-and-Diet groups (D).

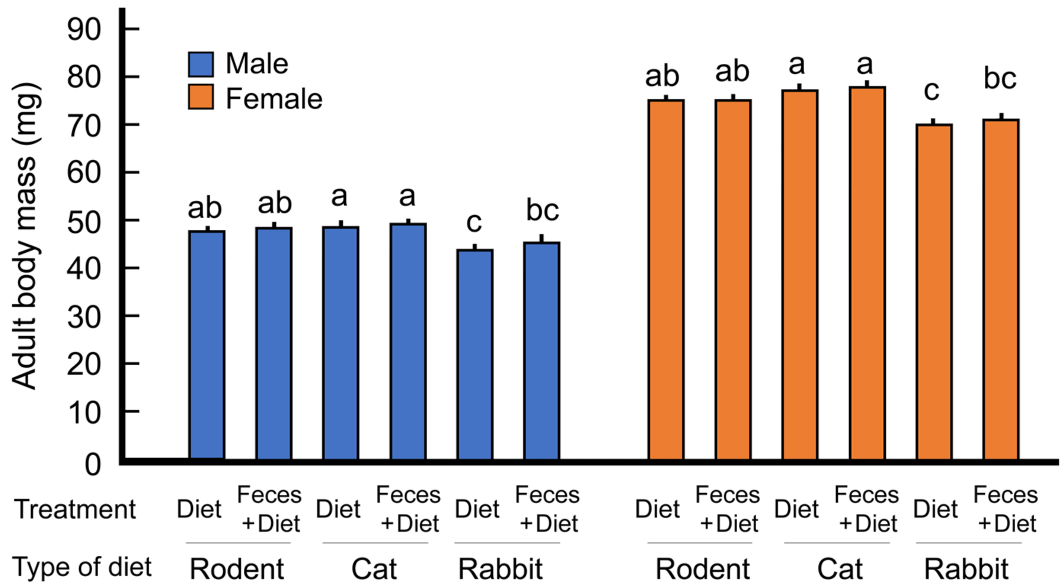


Figure 2. Adult body mass for Diet-only and Feces (from Rodent diet)-and-Diet groups. Adult male and female body mass (mean ± SEM) for cockroaches fed different diets in the Diet-only and Feces (from Rodent diet)-and-Diet groups. Different letters above the bars indicate significant differences among the treatment groups (ANOVA and Tukey's HSD, $p < 0.05$) for males and females separately.

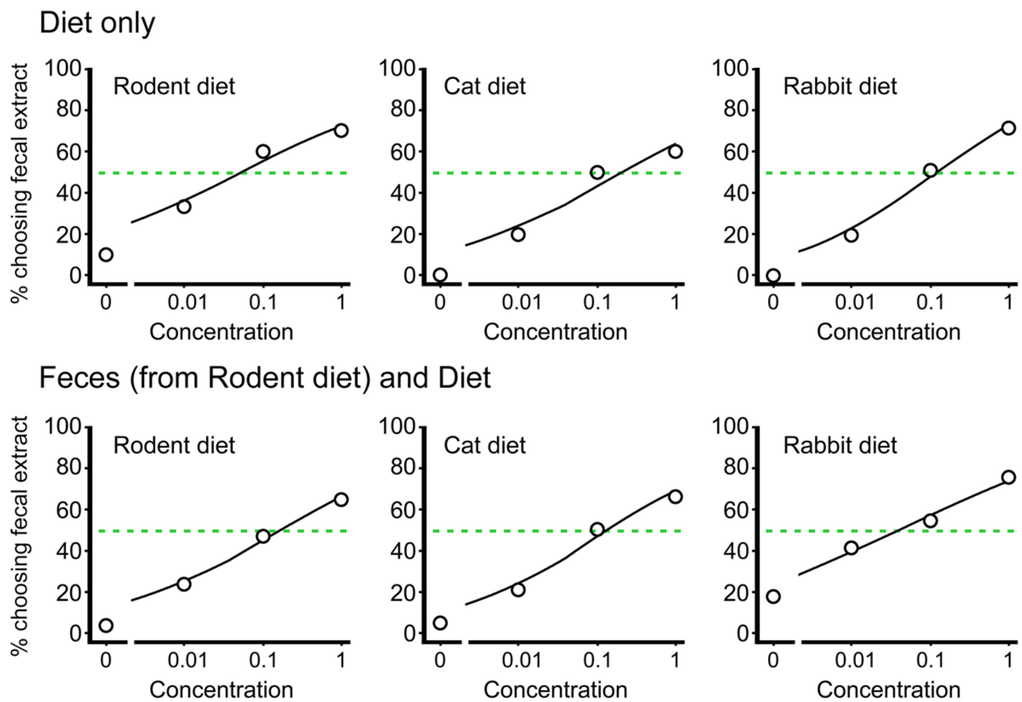
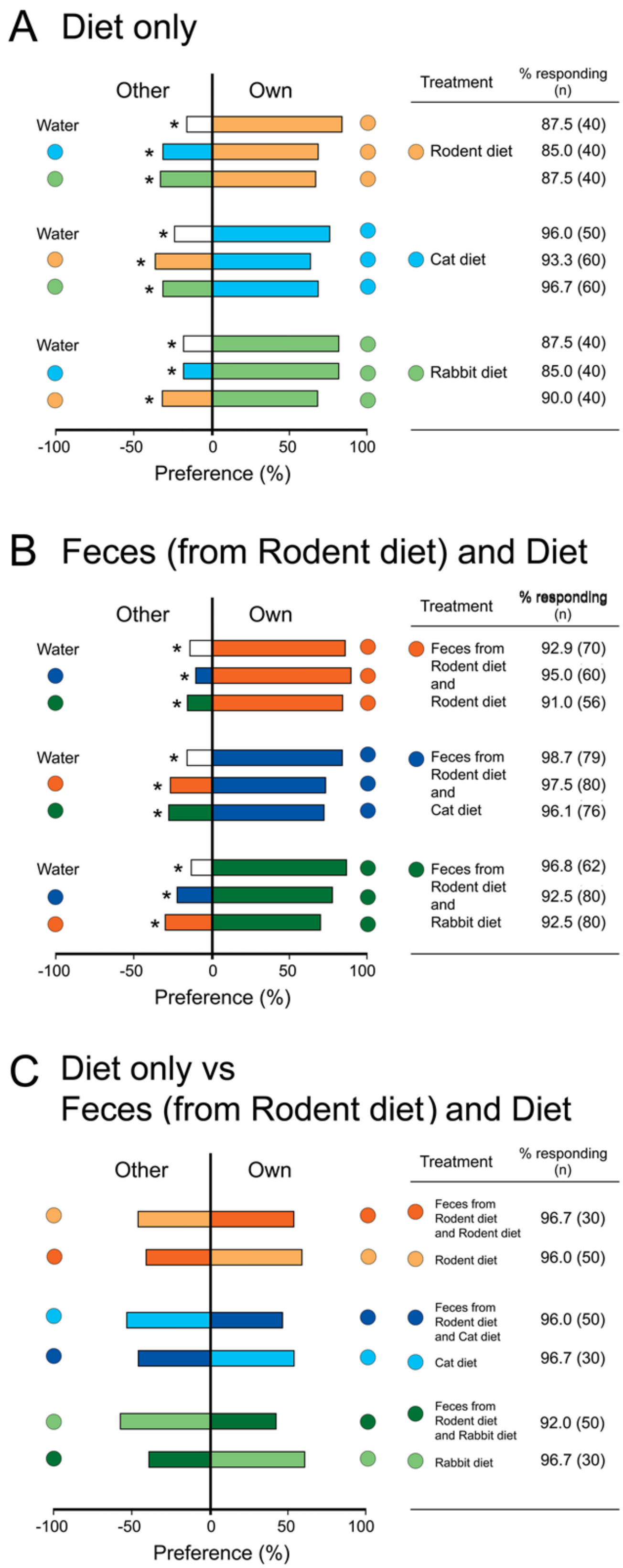


Figure 3. Dose-response curves for Diet-only and Feces (from Rodent diet)-and-Diet groups. First instar nymphs were given a choice between two filter paper shelters, one treated with sterile water and the other treated with fecal extracts from their own colony at three different concentrations: concentration 1 (water extract of 50 µg feces applied to filter paper in 1 µL), concentration 0.1 (water extract of 5 µg feces applied to filter paper in 1 µL), and concentration 0.01 (water extract of 0.5 µg feces applied to filter paper in 1 µL 0.5). The effective concentrations (EC_{50}) for nymphs fed different diets in each group are represented with a dashed line.

Figure 4. Aggregation preferences of nymphs in Diet-only and Feces (from Rodent diet)-and-Diet groups. Aggregation preferences of first instar nymphs in the (A) Diet-only and (B) Feces (from Rodent diet)-and-Diet groups given a choice between filter paper shelters treated with fecal extracts from their own colony or fecal extracts from a different colony, and (C) aggregation preferences of first instar nymphs in the Diet-only and Feces (from Rodent diet)-and-Diet groups given a choice between filter paper shelter treated with fecal extracts from cockroaches fed the same diet in the other experimental group. Different colored bars represent different treatment groups. Asterisks indicate a significant preference for a fecal extract (Chi-square test, $p < 0.05$).


A Diet only
Other
Own
Water
Treatment
% responding (n)
87.5 (40)
Rodent diet
85.0 (40)
87.5 (40)
96.0 (50)
Cat diet
93.3 (60)
96.7 (60)
87.5 (40)
Rabbit diet
85.0 (40)
90.0 (40)
-100 -50 0 50 100
Preference (%)
B Feces (from Rodent diet) and Diet
Feces from Rodent diet and Rodent diet
92.9 (70)
95.0 (60)
91.0 (56)
Feces from Rodent diet and Cat diet
98.7 (79)
97.5 (80)
96.1 (76)
Feces from Rodent diet and Rabbit diet
96.8 (62)
92.5 (80)
92.5 (80)
C Diet only vs Feces (from Rodent diet) and Diet
Feces from Rodent diet and Rodent diet
96.7 (30)
Rodent diet
96.0 (50)
Feces from Rodent diet and Cat diet
96.0 (50)
Cat diet
96.7 (30)
Feces from Rodent diet and Rabbit diet
92.0 (50)
Rabbit diet
96.7 (30)

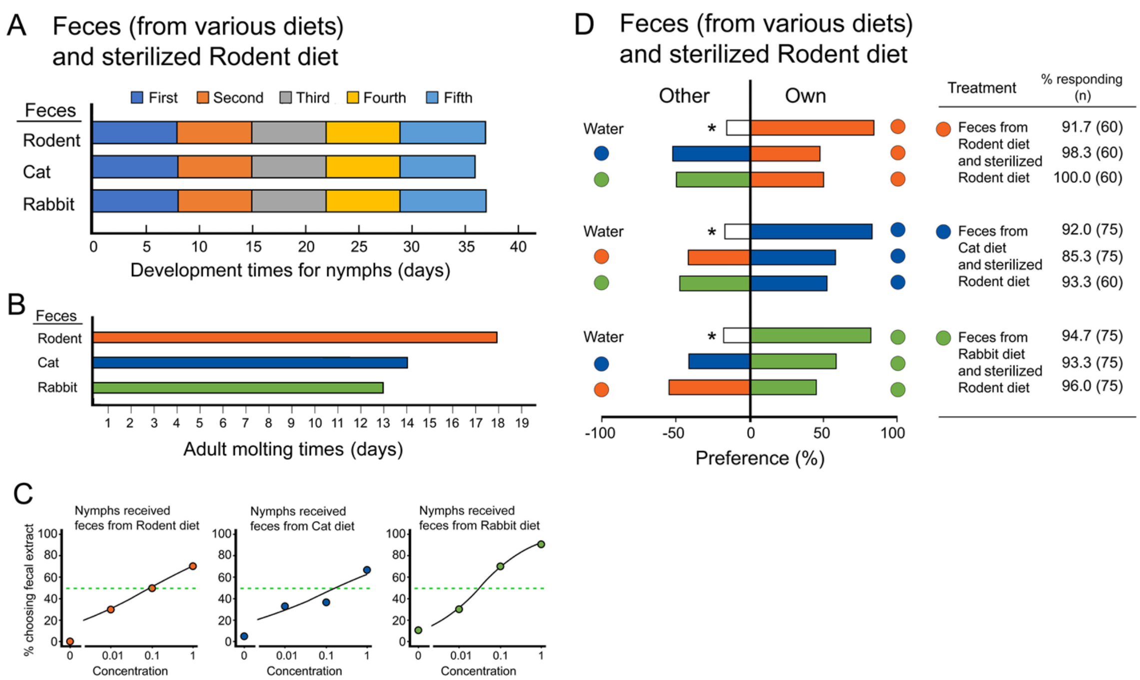


Figure 5. Development periods and aggregation preferences of nymphs in the Feces (from various diets)-and-Sterilized Rodent diet group. (A) Average number of days nymphs fed feces from cockroaches fed different diets spent in each instar stadium. Different colored bars represent different nymphal stages. (B) Average number of days it took nymphs fed feces from cockroaches fed different diets to become adults. (C) Results from dose-response assays using first instar nymphs fed feces from cockroaches fed different diets. Nymphs were given a choice between two filter paper shelters, one treated with sterile water and the other treated with fecal extracts from their own colony at three different concentrations: concentration 1 (water extract of 50 µg feces applied to filter paper in 1 µL), concentration 0.1 (water extract of 5 µg feces applied to filter paper in 1 µL), and concentration 0.01 (water extract of 0.5 µg feces applied to filter paper in 1 µL 0.5). The effective concentrations (EC_{50}) for nymphs fed feces from cockroaches fed different diets are represented with a dashed line. (D) Aggregation preferences of first instar nymphs fed feces from cockroaches fed different diets given a choice between filter paper shelters treated with fecal extracts from their own colony or fecal extracts from a different colony. Different colored bars represent different treatment groups. Asterisks indicate a significant preference for a fecal extract (Chi-square test, $p < 0.05$)

CHAPTER 4

Cockroaches as Trojan Horses for Systemic Control of Cockroach Aggregations with Baits

This work is formatted to be submitted to *Journal of Economic Entomology.*

Abstract

Gel bait formulations of insecticides have been shown to be highly effective in managing German cockroach (*Blattella germanica* L. [Blattodea: Ectobiidae]) populations. Two potential reasons for this are the use of slow-acting insecticides, and their systemic horizontal transfer within aggregations, a phenomenon known as 'secondary kill'. Our objective was to determine whether horizontal transfer can go beyond secondary, to tertiary and quaternary effects, and to compare various gel baits with different active ingredients. We fed adult females a gel bait and recorded their knockdown and mortality. Groups of first instar nymphs were then exposed to the feces and dead bodies of these females, secondary mortality was quantified, and a new cohort of nymphs was then exposed to the feces and dead nymphs (for tertiary mortality); this process was repeated for quaternary mortality. This design did not distinguish among the major mechanisms of horizontal transfer of insecticides: coprophagy and contact with feces, exposure to regurgitated fluids (emetophagy and contact) and cannibalism and necrophagy of nymphs. All the tested baits caused 100% mortality of the adult females that directly fed on the bait and relatively high secondary mortality (average of >85%) within 48 hrs. Baits containing dinotefuran, emamectin benzoate, fipronil or indoxacarb caused tertiary mortality (average of 15–70%), but only the fipronil and indoxacarb baits caused some, albeit low quaternary mortality (average of <15%) within 48 hrs. The relative importance of secondary, tertiary and quaternary transfer of the active ingredient remains to be determined in field populations of the German cockroach.

Key words: systemic insecticide, secondary mortality, tertiary mortality, quaternary mortality, horizontal transfer, gel bait

Introduction

Gel bait formulations of insecticides have revolutionized urban pest management, especially interventions to eliminate indoor infestations of cockroaches. Compared to residual sprays, baits offer greater efficacy, greater specificity, reduced non-target hazards, greater assortment of active ingredients with diverse modes of action, and utility in insecticide-sensitive areas (Buczkowski et al. 2001, Appel 2003, Gondhalekar et al. 2011, Schal 2011). Another reason that baits are highly effective is often attributed to their systemic translocation to aggregations where they can cause mortality of individuals that did not visit the bait. Secondary mortality, also known as 'secondary kill,' results from the horizontal transfer of insecticides that occurs when an individual exposed to an insecticide passes the toxicant to other individuals in the population, resulting in death (Silverman et al. 1991, Kopanic Jr and Schal 1997, Durier and Rivault 2000, Buczkowski and Schal 2001, Buczkowski et al. 2008). This often occurs when foraging individuals interact with an insecticidal bait or spray, and return to the nest or aggregation, where they contact others. Baits are particularly effective at causing secondary mortality because, compared to spray formulations, large amounts of active ingredient are ingested, and depending on the insecticide, large amounts of undigested insecticide are often defecated.

Secondary mortality was first reported in in the German cockroach, *Blattella germanica* L. (Blattodea: Ectobiidae), using radiolabeled hydramethylnon (Silverman et al. 1991). Four mechanisms have been shown to facilitate the horizontal transfer of insecticides in the German cockroach, including contact (Durier and Rivault 2000, Buczkowski and Schal 2001), coprophagy (Silverman et al. 1991, Kopanic Jr and Schal 1997, 1999, Buczkowski et al. 2001), emetophagy

(Buczkowski and Schal 2001), and necrophagy and cannibalism (Gahlhoff Jr et al. 1999, le Patourel 2000, Buczkowski et al. 2008). Only one study reported the horizontal transfer of insecticide beyond secondary mortality. Buczkowski et al. (2008) offered Advion bait, which contained 0.6% indoxacarb, to an adult male, quantified secondary mortality of first instar nymphs exposed to the adult male, and then offered the dead nymphs to starved adult males; 81% of the males died (tertiary mortality) largely from eating the dead nymphs. Nymphs exposed to the dead adults had no significant quaternary mortality, highlighting the importance of necrophagy in this design.

The overall goal of our study was to quantify the amount of secondary, tertiary, and quaternary mortality obtained from various cockroach gel baits. We hypothesized that all the baits would cause secondary mortality and that the amount of tertiary mortality would vary depending on the type and quantity of active ingredient in the bait. We also hypothesized that there would be low quaternary mortality among the baits. To minimize the impact of cannibalism and necrophagy and instead highlight the role of coprophagy, emetophagy and contact, we designed a sequence of experiments that examined the transfer of insecticide only among first instar nymphs.

Materials and Methods

Cockroaches

An insecticide-susceptible strain of *B. germanica* (Orlando Normal = American Cyanamid, collected in a Florida apartment in 1947) was used in this experiment. Cockroaches were reared on food pellets (Purina 5001 Rodent Diet, PMI Nutrition International, St. Louis, MO, USA) and were provided water in cotton-stoppered vials. Cockroach colonies were maintained at 27 ± 1 °C, 40–70% relative humidity, and L:D = 12:12 h photoperiod. Adult females were used to determine primary mortality and first instar nymphs were used to determine secondary, tertiary, and quaternary mortality. To promote feeding, all cockroaches were starved 24 hrs before being used in assays.

Insecticide Baits

The six commercial gel baits used in these experiments and their active ingredients are listed in Table 1. All baits were purchased from a local distributor.

Marking Cockroach Nymphs

To determine tertiary and quaternary mortality, our design (below) required first instar nymph donors to interact with first instar nymph recipients. Although genetic markers (e.g., orange-body) could be used to distinguish these two groups of nymphs, we marked the new first instar nymphs with a blue dye. Groups of first instar nymphs were starved for 24 hrs and allowed to drink blue-colored sugar water (0.5 mM erioglaucine disodium salt in 1 M sucrose) for 2 hrs before being introduced into the assay. The ingested blue-colored water could be seen in the nymph's gut for more than 4 hrs. When the blue-colored nymphs were provided non-dyed water, they would lose their blue coloring and a new group of blue-colored nymphs could be added to the assay in the next step.

Experimental Design

The design for this experiment is illustrated in Fig. 1. All assays were performed in glass jars (88 mm diameter, 95 mm height) the inner walls of which were coated with petroleum jelly and covered with cheesecloth secured with rubber bands. Ten adult females (3–4 days old) were placed in glass jar #1 along with ~0.5 g of fresh pre-weighed bait in a petri dish and water in a microcentrifuge tube. They were kept in this jar until all the females were dead; mortality was recorded as primary mortality at 0.5, 1, 3, 6, 12, 24, 48, 72, and 96 hrs. The bait was removed and 20 blue-colored nymphs were then added to jar #1, which also contained the dead females and any feces and residues from the dying cockroaches. The water was removed for the 4 hr assay so that nymphs would retain their blue color. The nymphs were then transferred to a new, clean glass jar (jar #2) with water and kept in this jar for 44 hrs. The number of dead nymphs was recorded as secondary mortality. Twenty new blue-colored nymphs were added into jar #2 with no water for 4 hrs. The nymphs were then transferred to a new, clean glass jar (jar #3) with water and were kept in this jar for 44 hrs. The number of dead nymphs were recorded as tertiary mortality. This process was repeated until there was no mortality in the jar. There were 5 replicates per treatment.

Statistical Analysis

Data analyses were performed using JMP Pro 14.2.0 software (SAS Institute 2019). Probit analysis was performed using PoloPlus (LeOra Software Company, Petaluma, CA) to compare knockdown times and mortality of adult females fed various baits. One-way analysis of variance (ANOVA) was used to separately compare secondary, tertiary, and quaternary mortality among the baits. Each ANOVA was followed by Tukey-Kramer's honestly significant difference (HSD) test ($\alpha = 0.05$) to identify differences among baits.

Results

Primary Knockdown and Mortality

All gel baits caused 100% mortality in adult females that fed on the baits and there was no mortality in the rodent diet control group. Baits containing dinotefuran caused 100% mortality within 6 hrs, clothianidin baits caused 100% mortality in 96 hrs, fipronil baits caused 100% mortality in 24 hrs, and indoxacarb baits, baits containing emamectin benzoate and baits containing clothianidin and pyriproxyfen caused 100% morality in 96 hrs (Fig. 2B). There were significant differences in the estimated LT_{50} and LT_{90} times of adult females among some of the treatment groups, based on non-overlap of 95% CIs (Table 1).

We also examined knockdown of adult females, because bait consumption would cease closer to knockdown than mortality. Most females were moribund within 24 hrs (Fig. 2A), suggesting that bait consumption would be rare beyond 24 hrs. The KT_{90} times are listed, along with the LT_{90} times, in Fig. 2B. Only ~20% of the females that fed on Optigard (emamectin benzoate) persisted beyond 24 hrs.

Secondary, Tertiary, and Quaternary Mortality

There was relatively high secondary mortality among the baits (Fig. 3A). All first instar nymphs exposed to adult females that fed on dinotefuran- or fipronil-containing baits died within 48 hrs. Secondary mortality of first instars on other baits ranged from 85% to 92%. There was no mortality in the control groups of first instars exposed to females that fed on rodent diet. Thus, all the baits caused significantly more secondary mortality than the control (Tukey-Kramer HSD test, $p < 0.0001$), but there were no significant differences among the six baits.

Tertiary mortality was defined as mortality in the second cohort of first instars that were exposed to the first cohort of first instar nymphs that were previously exposed to adult females that fed on a bait and died (Fig. 1). The level of tertiary mortality varied across the six baits (Fig. 3B). On average, the fipronil bait caused 70% tertiary mortality of first instar nymphs, indoxacarb bait and emamectin benzoate bait caused 34% and 32% mortality, respectively, and the dinotefuran bait caused 15% of the nymphs to die. There was no tertiary mortality in the clothianidin bait treatment nor in the Vendetta Nitro bait that contained both clothianidin and pyriproxyfen. All the nymphs in the control group survived. The fipronil bait caused significantly more tertiary mortality than any of the other baits (Tukey-Kramer HSD test; $p < 0.05$). Tertiary mortality was significantly higher in the fipronil, indoxacarb, and emamectin benzoate baits than in the rodent diet controls (Tukey-Kramer HSD test, $p < 0.05$) (Fig. 3B).

There was relatively low quaternary mortality in all bait treatments (Fig. 3C), with only 15.0 ± 5.2% mortality in the indoxacarb bait treatment, and 5.0 ± 2.7% in the fipronil bait treatment. There was no quaternary mortality in the other bait treatments and the rodent diet control. The indoxacarb bait caused significantly more quaternary mortality than the control and the other baits (Tukey-Kramer HSD test, $p < 0.001$), except the fipronil bait ($p = 0.0511$).

Discussion

Experimental Design

The experimental design of this study is the first of its kind used to investigate the transfer of insecticides within cockroach aggregations. It was used to maximize the amount of insecticide-contaminated material (feces, regurgitated liquids, and dead bodies) that cockroaches could contact, and focused on interactions between adults and nymphs and between groups of nymphs. German cockroach females were offered various baits (primary mortality) and used to deliver active ingredient to nymphs. Non-gravid females tend to eat more than males (Cochran 1983, Silverman 1986, Hamilton and Schal 1988), so that more feces would be available for nymphs to contact and eat (Silverman et al. 1991). We used first instar nymphs as targets of secondary, tertiary, and quaternary mortality, because they forage less frequently than other stages (Sommer 1975, Cloarec and Rivault 1991, Demark et al. 1993), and readily ingest adult excretions (Kopanic Jr and Schal 1997, Buczkowski and Schal 2001), increasing their exposure to insecticides.

Our design was substantially different from a previous study using indoxacarb bait, where tertiary mortality was measured by allowing adult males to contact and feed on dead nymphs for 72 hrs, highlighting the role of necrophagy (Buczkowski et al. 2008). The latter design showed significant tertiary mortality compared to the control (no mortality), but no quaternary mortality. We demonstrated mortality at the secondary, tertiary, and quaternary levels for different gel baits. Overall, we found that all baits caused 100% primary mortality of the adult females and an average of ≥85% secondary mortality of first instar nymphs. Four of the baits caused tertiary mortality at an average level of 15–70%. Only two of these baits resulted in quaternary mortality, with an average mortality of ≤15%.

It is important to note that these and related experiments were conducted in the laboratory in small arenas that maximized contact with the insecticides. A major caveat is that the processes and mechanisms we discuss below could be different in larger arenas and in the field.

Mechanisms of Horizontal Transfer of Insecticides

Association of neonates with older conspecifics is common in cockroaches, and it ranges from short-term contact with the mother (brooding), to long term membership in aggregations (Gautier et al. 1988, Nalepa and Bell 1997, Nalepa et al. 2001). Four major non-mutually exclusive mechanisms have been shown to facilitate the horizontal transfer of insecticides in German cockroach aggregations: ingestion of feces (coprophagy) and various other excretions (e.g., emetophagy), contact with dead or dying cockroaches and their excretions, necrophagy, and cannibalism.

Cannibalism is not common in cockroaches and tends to occur under stressful conditions, such as elevated temperature, high population density, and limited resources (Guthrie and Tindall 1968). Some cannibalism may occur in all stages and both sexes of the German cockroach even in the presence of sufficient food, water, and harborage (Aparicio 1996), but it is not a common occurrence in this species. Nevertheless, cannibalism can be a major factor in a carefully manipulated experimental design – starved adult males readily cannibalized indoxacarb-intoxicated first instars, resulting in tertiary transfer of the insecticide and significant mortality (Buczkowski et al. 2008).

Necrophagy appears to be important in the nutritional ecology of cockroaches, with crop contents of cockroaches in natural forest populations found to contain arthropod cuticle (Schal et al. 1984), presumably from scavenged dead insects and related organisms (Gahlhoff Jr et al. 1999). Necrophagy occurs with both fast- and slow- acting insecticides and various life stages (Gahlhoff Jr et al. 1999, Durier and Rivault 2000).

Coprophagy is common in insects and it offers two major adaptive benefits: (a) inoculation with mutualistic microbes that assist in digestion, immune responses and behavior, and (b) nutritional benefits from ingesting undigested food, enzymes, and other metabolites (Stevenson and Dindal 1987, Nalepa et al. 2001, Weiss 2006). Coprophagy and emetophagy have been documented as prominent mechanisms in horizontal transfer of baits in cockroaches. Using radiolabeled hydramethylnon, Silverman et al. (1991) documented that nymphs readily ingest the feces of adults that fed on hydramethylnon-containing baits. Other studies documented the importance of coprophagy in both small and large (>1 m) arenas using various exclusion methods to prevent either adults or first instars from directly consuming the bait (Kopanic Jr and Schal 1999). Coprophagy was shown to be especially adaptive to first instar nymphs that forage less and can develop into second instars solely on adult feces (Kopanic et al. 2001). Nevertheless, the relative importance of coprophagy varied across different baits with various active ingredients (Silverman et al. 1991, Kopanic Jr and Schal 1997, 1999, Buczkowski et al. 2001). Emetophagy was first reported with fipronil, where cockroaches that ingested bait were found to exude liquid excretions that appeared attractive and lethal to conspecifics within the aggregation (Buczkowski and Schal 2001). This behavior could also occur with other neurotoxic insecticides, including relatively slow-acting indoxacarb (Buczkowski et al. 2008).

Many experimental designs, including ours, cannot differentiate among these various mechanisms of horizontal transfer of insecticides. Cannibalism and necrophagy are often difficult to separate without real-time monitoring (e.g., video), and emetophagy likewise requires careful real-time observations because the secretions of dying individuals quickly dry out. Contact is a component of all these mechanisms, and its relevance in insecticide transfer depends largely on characteristics of the active ingredient. For example, systemic insecticides like hydramethylnon have little activity through contact, while most neurotoxic insecticides are effective by contact. Thus, in some cases, the presence of dead contaminated cockroaches caused mortality mainly through contact rather than by necrophagy (Durier and Rivault 2000).

In our experimental design, the horizontal transfer of insecticides could occur through contact or ingestion of feces and regurgitated fluids (coprophagy and emetophagy), and direct contact with contaminated individuals. Necrophagy and cannibalism were much less likely in aggregations of first instar nymphs. While coprophagy had an impact on secondary mortality when nymphs were able to feed on adult female feces, the prominence of this behavior in tertiary and quaternary mortality remains to be determined.

Properties of Systemic Insecticides and their Effects on Horizontal Transfer

Effective bait formulations must be palatable and not deterrent, attractive and not repellent, easy to consume, and toxic in the amounts consumed (Appel 1990, Reierson 1995). Although fast-acting contact insecticides are generally thought to be more repellent than slow-acting toxicants (Ebeling et al. 1966), and the latter provide greater cockroach population reductions in field trials (Milio et al. 1986, Appel 1992, Appel and Benson 1995), this generalization appears to have been made for pyrethroids and older insecticide classes that are no longer used in indoor baits. Fast-acting insecticides used in modern bait formulations are often highly palatable and not repellent. In most investigations of secondary kill, including ours, repellency likely played a minor role, if any, because cockroaches are usually restricted to small arenas and starved before the assay begins to maximize bait consumption. Therefore, assuming high relative attraction to the bait, three major features of baits would significantly affect secondary kill under these conditions: palatability and amount of bait ingested, transit time of the bait and its detoxification in the digestive system, and the speed-of-action of the insecticide. By starving adult females for 24 hrs, we maximized ingestion of all baits. Further, it is reasonable to assume that large amounts of all the baits were consumed, independently of the insecticide's speed of action, because bait intake likely occurred in minutes, before the onset of symptoms from fast-acting insecticides.

The transit time through the gut and the insecticide's speed of action are closely related. Relatively faster-acting insecticides might compromise secondary mortality for two reasons. First, because the intoxicated cockroaches might not be able to return to the harborage after they ingested the bait (Stejskal et al. 2004). Although this might be a significant concern in real-world situations and in large arenas, return to the harborage was not a component of our assays in small containers. Second, fast-acting insecticides might kill the insect before the bait transits through the alimentary canal, and thus compromise secondary mortality through coprophagy. For example, the transfer of insecticides through coprophagy was less effective with fast-acting insecticide baits that failed to traverse the alimentary tract before the forager died (Kopanic Jr and Schal 1999), but was effective with delayed action insecticides like hydramethylnon (Silverman et al. 1991, Buczkowski and Schal 2001). Thus, residues of hydramethylnon killed most nymphs and adults in secondary mortality assays, whereas residues of fast-acting insecticides (like chlorpyrifos and fipronil) killed fewer cockroaches (Buczkowski et al. 2001).

In our assays, all six baits caused significant secondary mortality, but the level of tertiary and quaternary mortality varied with the modes of action and speed of the active ingredients. Alpine bait (dinotefuran), which had rapid KT_{90} and LT_{90} of <0.5 and 2 hrs, respectively, resulted in low tertiary mortality, consistent with the idea that fast-acting insecticides are less effective in horizontal transfer. On the other hand, however, Maxforce FC (fipronil), also a fast-acting insecticide (KT_{90} and LT_{90} of <3 and 7.5 hrs, respectively), had the highest tertiary mortality among the six baits. Clothianidin-containing baits (Vendetta Nitro and Maxforce Impact), which were slower-acting insecticides (KT_{90} and LT_{90} of <3 and <31 hrs, respectively), presumably giving females more time to eat and defecate, resulted in no discernible tertiary mortality. Finally, the slowest acting baits, Optigard (emamectin benzoate) and Advion (indoxacarb), with KT_{90} values of 34 and 14 hrs and LT_{90} values of 42 and 67 hrs, respectively, resulted in similar levels of intermediate tertiary mortality; Advion, however, had the highest quaternary mortality of any bait. Overall, these results suggest a complex relationship between the speed-of-kill of an insecticide and its horizontal transfer. In assays in small containers, as in our assays, foraging distance is minimal, and transit time of the bait through the digestive system is probably more important than other considerations. However, insecticides with exceptionally low LD_{50} by both contact and ingestion (e.g., Maxforce FC [fipronil]) (Ko et al. 2016) can be effectively transferred among cockroaches and cause secondary mortality and beyond, even if they are relatively fast acting. Assuming 5 mg of Maxforce FC bait ingested, each adult female acquired ~2.5 µg of fipronil, which is almost 2,000-fold the topical LD_{50} of an adult male (Gondhalekar et al. 2011). Similar extrapolations with indoxacarb (Advion), the slowest of the insecticides we tested, estimate ~30 µg of indoxacarb per female, which is 375-fold the topical LD_{50} of an adult male (Gondhalekar et al. 2011). Presumably, substantial amounts of fipronil or indoxacarb would be available in feces. Although these two insecticide baits differ in various features, they were nevertheless highly effective in our tertiary and quaternary assays with insecticide-susceptible cockroaches.

Perspectives

Two related topics need to be considered in future investigations to understand the magnitude and significance of horizontal transfer of baits among cockroaches – there is a dire need to quantify secondary mortality in residential settings, and to consider how insecticide resistance might impede this process. Thus far, the only attempt to estimate secondary mortality in the field was a study of lab-reared cockroaches released into a vacant apartment. Using a reciprocal exclusion design, whereby adults or first instars were prevented from ingesting insecticide bait, Kopanic Jr and Schal (1999) demonstrated that horizontal transfer of insecticide to nymphs played a significant role in neonate mortality. We suspect that secondary effects might be substantial in the field, because small nymphs forage only short distances, and the relative magnitude of horizontal transfer of bait insecticides (compared to direct ingestion of bait) is positively related to distance of the bait from the aggregation (Silverman 1986, Kopanic Jr and Schal 1999). Unfortunately, thus far there has not been a satisfactory method to quantify secondary effects in the field. A promising approach could be to use UV light to track insecticide-laden feces. Laboratory experiments revealed that fluorescent bait was accepted by cockroaches as a food source, feces produced after its consumption were also fluorescent, and consumption of fluorescent bait led to continuous fluorescent feces production, giving cockroaches enough time to deposit fluorescent feces within aggregations (Varadinova et al. 2015).

The transfer of insecticide from foragers to first instars undergoes a considerable dilution factor, in part related to insecticide degradation in the digestive system. Thus, high levels of insecticide resistance might lessen the significance of insecticide transfer within aggregations. Ko et al. (2016) used nymphs of two color morphs to distinguish unselected insecticide-susceptible nymphs from insecticide-resistant nymphs; this design allowed both strains to co-habit the same arena, which equally exposed them to adult feces. Nymphs that were multiresistant to fipronil, indoxacarb, and hydramethylnon survived significantly longer than insecticide-susceptible nymphs on residues of bait-fed males, demonstrating that resistance significantly lessened secondary kill (Ko et al. 2016). These findings raise an important question: Does the translocation of active ingredients expose cockroaches to moderate and sublethal doses that could accelerate the evolution of insecticide resistance and thus negate the benefits of secondary kill in population suppression?

Overall, the level of secondary mortality that can be attained with different insecticides will depend on many factors, including concentration of the active ingredient in the bait, stability of the active ingredient in the insect's digestive tract, relative toxicity of the active ingredient, amount of the active ingredient excreted, amount of the active ingredient bioavailable after excretion, mechanism of translocation, and insecticide resistance (Buczkowski and Schal 2001). By understanding how these factors interact and by designing baits that incorporate life history traits and adaptations of the German cockroach, we can potentially develop gel baits that can attain higher levels of mortality to suppress cockroach populations.

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Table 1. Time-course of mortality of *B. germanica* adult females after consuming various baits.

Bait (% active ingredient)	Manufacturer	*n*	LT_{50} hrs (95% CI)[a]	LT_{90} hrs (95% CI)[a]	Slope ± SEM	χ^2 (df)	*t*-ratio[b]
Rodent diet (control)	NA	100	NA	NA	NA	NA	NA
Alpine (0.5% dinotefuran)	BASF	100	0.91 (0.83–1.00)	1.97 (1.71–2.36)	3.84 ± 0.37	0.42 (2)	10.32*
Maxforce FC (0.05% fipronil)	Bayer	100	3.63 (2.52–4.82)	7.51 (5.54–13.81)	4.06 ± 0.34	26.66 (4)	12.01*
Maxforce Impact (1.0% clothianidin)	Bayer	100	4.60 (3.11–6.49)	23.75 (15.42–45.64)	1.79 ± 0.10	34.25 (6)	17.07*
Vendetta Nitro (0.5% clothianidin)	MGK	100	4.83 (3.48–6.49)	30.91 (21.29–51.24)	1.59 ± 0.09	27.26 (7)	18.08*
Optigard (0.1% emamectin benzoate)	Syngenta	100	19.53 (17.07–22.22)	42.47 (36.23–52.05)	3.80 ± 0.24	14.61 (7)	15.50*
Advion (0.6% indoxacarb)	Syngenta	100	26.76 (22.32–31.83)	66.80 (53.71–90.32)	3.23 ± 0.20	24.24 (7)	16.13*

[a] The LT_{50} and LT_{90} represent the time (hrs) for 50% and 90%, respectively, of the 100 females in each treatment to die. CI = confidence interval. NA = not applicable.

[b] *t*-ratio of the slope. Values >1.96 denote a significant regressions (* $P < 0.05$).

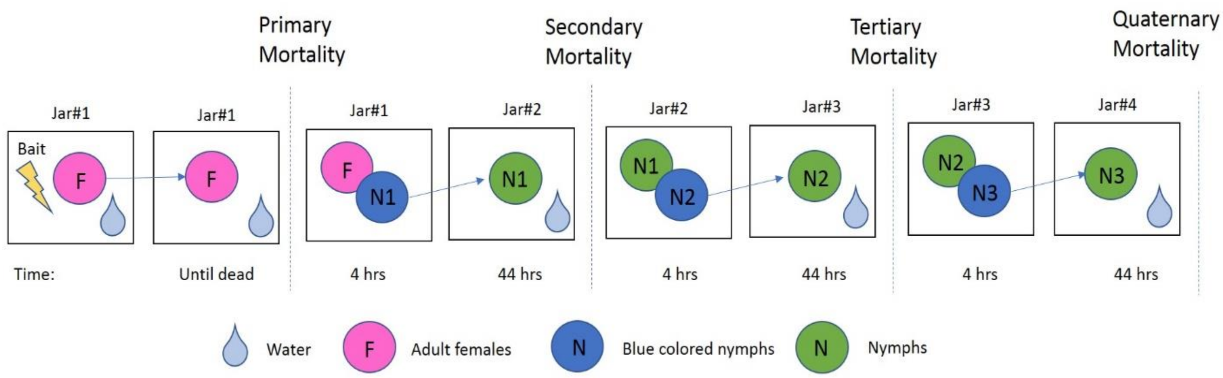

Figure 1. Experimental design demonstrating the horizontal transfer of insecticides. Diagram of the experimental design illustrating primary mortality in adult females fed various gel baits, the horizontal transfer of insecticides from adult females to nymphs (secondary mortality) and from nymphs to nymphs (tertiary and quaternary mortality). No-choice assays were performed in a series of glass jars. Ten adult females fed on a bait, the bait was removed once all the females were dead, and primary mortality was recorded. A group of twenty blue-dyed first instar nymphs was added to the same jar (jar #1) for 4 hrs and then moved to a new, clean glass jar (jar #2) and secondary mortality was recorded after 44 hrs. By this time, the nymphs had lost their color and a new group of twenty blue-dyed first instar nymphs were added to jar #2 for 4 hrs. The new nymphs were moved to jar #3 and tertiary mortality was recorded 44 hrs later. The process of adding twenty blue-dyed nymphs to the jar for 4 hrs, then moving them to a new jar and recording mortality after 44 hrs was repeated until there was no mortality. There were 5 replicates per treatment.

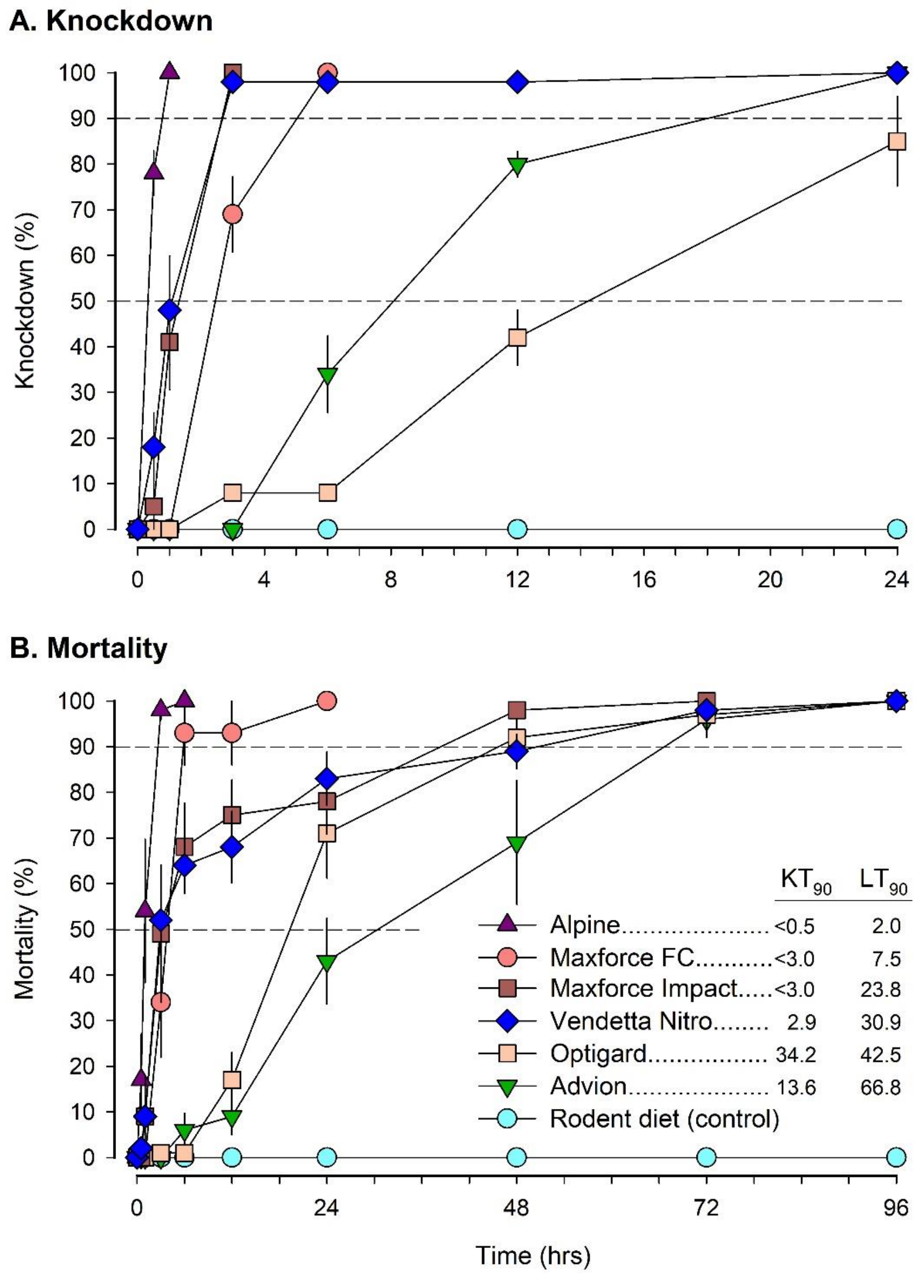

Figure 2. Primary Knockdown and mortality of adult females. Time-course of mean (A) knockdown (± SEM) over 24 hrs and (B) mortality (± SEM) over 96 hrs of adult females feeding on gel baits or rodent diet (control). There were 5 replicates per treatment (*n* = 20 females per replicate). Knockdown and mortality were subjected to probit analysis, and KT_{90} and LT_{90} values are shown in (B). Dashed lines show the 50% and 90% levels for both knockdown and mortality.

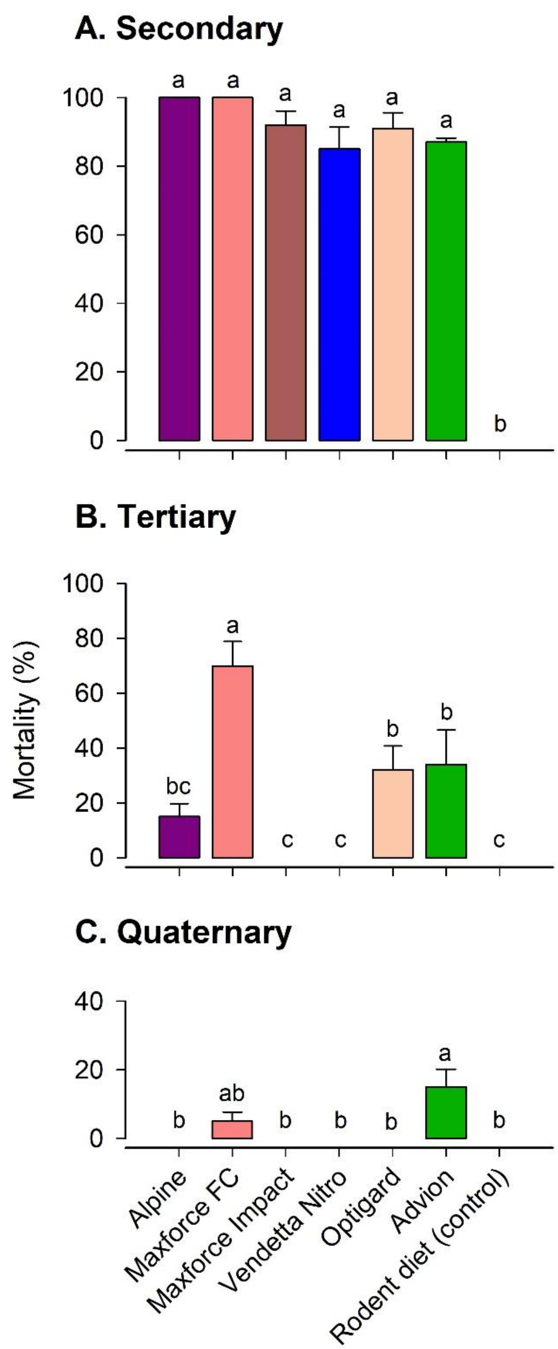

Figure 3. Mortality of first instar nymphs. Mean (± SEM) percent (A) secondary, (B) tertiary, and (C) quaternary mortality of first instar nymphs. Each bar represents a different gel bait (n = 20 nymphs per replicate, 5 replicates per treatment). Different letters above the bars indicate significant differences among the treatments within each panel (ANOVA, Tukey-Kramer's HSD test, $p < 0.05$)**.**

CHAPTER 5

Effects of novaluron ingestion and topical application on German cockroach (*Blattella germanica*) development and reproduction

(This work was published in *Pest Management Science*: **Hamilton, J. A., Wada-Katsumata, A., Ko, A. and Schal, C.** 2021. Effects of novaluron ingestion and topical application on German cockroach (*Blattella germanica*) development and reproduction. *Pest Management Science* 77: 877–885. doi: 10.1002/ps.6092)

Abstract

BACKGROUND: Insect growth regulators (IGRs) disrupt insect development and reproduction. Chitin synthesis inhibitors (CSIs) allow the insect to grow normally, but because chitin is an essential component of the cuticle, formation of a new cuticle and ecdysis are prevented and the insect dies. CSIs can also kill embryos by disrupting their normal development. We evaluated the potential utility of novaluron in bait formulations against the German cockroach (*Blattella germanica* L.).

RESULTS: The minimum novaluron intake that interfered with molting and reproduction was assessed by exposing nymphs and adult females to novaluron. Results showed that one day of feeding on 0.1% novaluron was sufficient to disrupt molting in nymphs and prevent adult females from developing viable oothecae. The long-term effects on gravid females were investigated by feeding females 0.1% novaluron for different 5-day intervals during successive stages of gestation. Results demonstrated that gravid females fed novaluron during any period of gestation were able to produce viable eggs. To determine if ingestion of novaluron affected mating success and fertility of adult males, males were fed novaluron and then allowed to mate with untreated virgin females. Males that fed on novaluron successfully mated, and the females produced viable oothecae. Finally, direct comparisons revealed that novaluron is equally effective by ingestion and topical application.

CONCUSIONS: Novaluron caused mortality in nymphs and interfered with ootheca production in adult females, but only before they formed an ootheca. It successfully reduced German cockroach populations in cages and has potential to be incorporated in cockroach baits.

Keywords: insect growth regulator; chitin synthesis inhibitor; novaluron; cockroach; reproduction; sterility

1 INTRODUCTION

Insect growth regulators (IGRs), also termed insect growth disruptors, have been used in pest management since the 1980s. These chemicals interfere with physiological and biochemical processes that are essential for normal growth, development, and reproduction in insects[1,2]. IGRs are compatible with integrated pest management (IPM) programs because they are specific to the target pest species, have minimal effects on beneficial insects, and have unique modes of action that are different from conventional broad-spectrum (neurotoxic) insecticides.[1-3] IGRs are grouped according to their mode of action, either as chemicals that mimic key insect hormones (juvenile hormone analogs [JHAs], ecdysone agonists), or interfere with cuticle formation (chitin synthesis inhibitors [CSIs]).

Several CSIs (all benzoylphenyl ureas [BPUs]), have been studied for their potential in cockroach management. Diflubenzuron,[4,5] penfluron,[4] triflumuron,[6-8] chlorfluazuron,[5,9] UC 84572,[5] flufenoxuron,[10] lufenuron,[11,12] and noviflumuron[13-15] have been shown to be effective in reducing German cockroach (*Blattella germanica* L.) populations[16]. They disrupt the deposition of chitin in the newly synthesized cuticle, causing the new exoskeleton to weaken, and resulting in nymphs dying during ecdysis.[4-7,9,11,12,17] In cockroach embryos, CSIs disrupt cuticle formation, which prevents hatching of neonates.[2,3,18]

Novaluron is also a BPU. The specific mode of action of novaluron is not well understood, but the general mechanisms are similar to other BPUs.[19] Novaluron has been used to manage various insect pests, including crop pests such as *Spodoptera littoralis*, *Bemisia tabaci*[20] and *Leptinotarsa decemlineata*,[21] structural pests (termites),[22,23] medically important mosquitoes,[24-27] and stored product beetle pests.[28] Novaluron has demonstrated activity primarily through ingestion, but was shown to disrupt ecdysis and interfere with embryogenesis by contact as well.[19] The US Environmental Protection Agency,[29] Canadian Pest Management Regulatory Agency,[30] Food and Agriculture Organization, and World Health Organization[31,32] consider novaluron a low risk to the environment and non-target organisms, and value it as an important option for IPM that should decrease reliance on organophosphate, carbamate and pyrethroid insecticides.

The overall goal of this project was to evaluate the potential utility of novaluron in bait formulations for management of German cockroach populations. We hypothesized that novaluron, like other CSIs, will cause death of nymphs during ecdysis and interfere with reproduction in adults. First, we assessed its effects on the structure of a mixed-stage cockroach population containing early and late instar nymphs and on the structure of a population containing only adult males and females. Next, we determined the minimal novaluron intake that would interfere with molting in nymphs and reproduction in adults. We then assessed the effects of novaluron ingestion by gravid females on their offspring and whether ingestion of novaluron by males affected their mating success and fertility. Finally, we conducted dose-response studies to directly compare the bioactivity of novaluron through ingestion and topical application.

2 MATERIALS AND METHODS

2.1 Cockroaches

The cockroaches used in these experiments were from a standard insecticide-susceptible strain of *B. germanica* (Orlando Normal = American Cyanamid, collected in a Florida apartment over

70 years ago). They were reared on food pellets (Purina 5001 Rodent Diet, PMI Nutrition International, St. Louis, MO, USA) and water was provisioned in cotton-stoppered vials. Cockroach colonies were maintained at 27 ± 1 °C, 40–70% relative humidity, and L:D = 12:12 h photoperiod.

2.2 Insect growth regulator

Novaluron-supplemented diets were prepared by adding the appropriate amount of novaluron (98.5%, Control Solutions-ADAMA, Pasadena, TX), dissolved in 20 ml of acetone, to 100 g of ground rodent chow. The slurry was thoroughly mixed, and acetone was allowed to evaporate from the novaluron-chow mix at least overnight. For most studies novaluron-containing diets were prepared at the following concentrations: 0% (acetone control), 0.0001, 0.001, 0.01, 0.025, 0.1, and 0.5% (1, 10, 100, 250, 1000 and 5000 ppm). For the comparison of ingested and topically applied novaluron, we used ground rodent chow that contained 0% (acetone control), 0.0002, 0.002, 0.02 and 0.2 (2, 20, 200, and 2000 ppm) novaluron. To generate small food particles, rodent chow in this experiment was ground in a micro-mill grinder with stainless steel blade and grinding chamber (Bel-Art, South Wayne, NJ).

2.3 Population studies

To test the effects of various novaluron concentrations on the demographic structure of German cockroach populations, no-choice and two-choice assays were conducted in rectangular cages (18.7 x 13.3 x 9.5 cm, T-79, Althor Products, Windsor Locks, CT, USA), each containing an egg carton shelter and a cotton-stoppered water vial. Adults and nymphs were separated in different cages to allow us to monitor adult emergence in cages of nymphs, and each concentration was tested separately in no-choice assays and two-choice assays. Each adult cage (3 replicates per treatment) contained 10 males (> 7 days old) and 10 females (0–2 days old). Each nymph cage (3 replicates per treatment) contained 50 early instars (first to third) and 30 late instars (fourth and fifth). Cockroaches were placed into the cages, acclimated for 24 h with untreated rodent chow, and then the rodent chow was replaced by the appropriate novaluron-containing diet. For no-choice tests, 1–2 g of the appropriate diet (0 to 0.5% novaluron) was placed in a disposable plastic Petri dish (5.5 cm diameter, Falcon-Corning, Corning, NY, USA) and replaced as needed. For two-choice assays, 1–2 g of each diet was placed in two Petri dishes positioned equal distance from the shelter and water. The treatments included acetone control diet (0% novaluron) vs. various concentrations of novaluron-supplemented diets. The 0% diet was also offered in a two-choice assay with untreated rodent chow (no acetone) to confirm that acetone did not affect the palatability of the diet. The demography of each cage (number in each stage alive) was recorded weekly.

One set of experiments (0.0001, 0.001, 0.01% novaluron) was conducted at 27 ± 1 °C. A second set of experiments (0, 0.025, 0.1 and 0.5% novaluron) inadvertently experienced a lower temperature, about 25 °C. Therefore, the general patterns may be compared across the full dose-response, but key developmental events (eclosion, ootheca formation, hatching) occurred sooner in the experiments conducted at 27 °C.

2.4 Minimal novaluron intake that interferes with molting in nymphs and reproduction in adults

To determine the minimal novaluron intake that would interfere with molting, no-choice assays were conducted with first instars. Groups of 30 newly hatched nymphs were separated into cages (T-29 Althor) with water, shelter, and 1 g of 0.1% novaluron-supplemented chow.

Nymphs were allowed to feed for 0 h (control group), 24, 48 or 72 h, and then the diet was replaced with untreated ground rodent chow. Mortality and molting success were recorded daily for 14 days. There were 4 replicates per treatment.

A similar experiment was conducted using adult females to determine the minimal novaluron intake that would interfere with reproduction. Groups of 10 adult females (newly eclosed 0 days old) were placed into cages (T-29 Althor) with water, shelter, and ground rodent chow. The females were allowed to feed on 1 g of 0.1% novaluron-supplemented chow for either 0 h (control group), 24, 48 or 72 h. The treated diet was then replaced with 1 g of untreated ground rodent chow and 10 adult males (2 weeks old, virgin) were added to each cage for mating. To ensure that females were exposed to treated diets during their maximal food consumption and allowed to mate at the same age, females were presented the diets at different ages. Thus, females in the 24 h group were given novaluron between days 4 and 5, females in the 48 h group were given novaluron between days 3 and 5, and females in the 72 h group were given novaluron between days 2 and 5. All females in all groups were 5 days old when males were added. The number of females that formed oothecae was recorded. The females were monitored for another 21 days to determine if the embryos were viable. There were 4 replicates per treatment.

2.5 Effects of novaluron on gravid females

Unlike most oviparous cockroaches (such as *Periplaneta americana* and *Supella longipalpa*), female *B. germanica* retain the ootheca for the entire period of embryogenesis. We tested whether novaluron ingested by females after ootheca formation would affect the maturing embryos. Groups of 20 adult females (5 days old) were placed in six cages (T-29 Althor) and 20–

22 adult males (8–15 days old) were added to each cage. Females were monitored daily and when an ootheca was visible (day 0), the female was placed singly in a disposable plastic Petri dish (9 mm diameter, Falcon-Corning) with 0.3 g rodent chow, water, and shelter. Individually housed females were placed into groups and each group was exposed to novaluron during a different 5-day interval during gestation. The rodent chow in the Petri dish was replaced with 0.1% novaluron-chow for a 5-day interval during gestation, as follows: no exposure to novaluron (control group), exposure to novaluron on days 0 to 5, 5 to 10, 10 to 15 and 15 to 20 post oviposition. We also included a group of gravid females exposed to novaluron for the entire gestation period, days 0 to 20. There were 20 individually housed females per group. Females were then monitored and deformed or aborted oothecae were noted. When a female aborted or hatched the ootheca, it was moved to a new Petri dish with untreated chow, water, shelter, and a fresh male (>8 days old). The males were removed when the female formed an ootheca and the females were again monitored for viability of the embryos within each ootheca.

2.6 Effects of novaluron on males

To investigate if novaluron affected mating success and fertility of males, groups of 50 newly emerged adult males were fed 0% chow (acetone-treated) or novaluron-chow (0.1% or 0.5%) for 13 days. Before placing them with females, males were fed untreated rodent chow for 24 h to minimize transfer of novaluron to females either on the cuticle or in male feces. Groups of 30–40 newly emerged adult females were fed rodent chow for 5 days, then paired individually with one male in a Petri dish (9 mm diameter) with untreated rodent chow and water. The pairs were video-recorded for 3 h in a temperature-controlled (27°C) room to confirm that mating occurred, and males were removed when an ootheca was formed. The phallomere of unmated males was inspected under a microscope for any abnormalities that might interfere with copulation. Females that formed an ootheca were kept in their individual Petri dishes and viability of the embryos within the oothecae was recorded.

2.7 Dose-mortality assays with ingested and topically applied novaluron

Newly emerged last instars (sex not determined) were housed in groups for 2 days with access to rodent chow and water. On day 3 they were placed in a 9 cm Petri dish with water in a cotton-stoppered microcentrifuge tube and starved for 24 hrs, and on day 4 each nymph was provided 5.0–5.2 mg of novaluron-supplemented ground rodent chow in a microcentrifuge tube cap. Nymphs rarely walked over the caps, which minimized the ground rodent chow from being displaced. Only nymphs that consumed all the novaluron-containing rodent chow were retained. Thus, the dose of novaluron ingested was estimated as the novaluron concentration per mg multiplied by 5 mg, so the novaluron doses we tested were 0, 0.01, 0.1, 1 and 10 µg. On day 5, each nymph was offered fresh rodent chow *ad libitum* and molting success was assessed daily.

For topical applications of novaluron, nymphs were treated as above, but on day 4 each nymph received two successive topical applications (0.5 µl each) of novaluron in acetone (0, 0.01, 0.1, 1 and 10 µg). Application was made between the mesothoracic coxae, an area that is least accessible to grooming to minimize the ingestion of groomed novaluron.

We conducted similar experiments with adult females, as follows: Newly eclosed females were fed rodent chow in groups, starved for 24 hrs on day 3, and offered 5 mg of novaluron-containing or control rodent chow. Some females received topical applications, as described for nymphs. On day 5 females were placed back in groups with fresh rodent chow, water and sexually mature males (14–30 days old). Females were monitored daily, and as each female formed an ootheca the female was separated in a Petri dish with food and water and monitored daily for hatching or abortion of the egg case.

2.8 Statistics

Kaplan-Meier analysis was performed using JMP[33] to compare mortality of small and large nymphs in no-choice and two-choice assays for each novaluron concentration, and to compare mortality of first instars fed 0.1% novaluron for 0 h (control group), 24, 48 or 72 h. The effect of 0.1% and 0.5% novaluron on mating success and ootheca hatch were analyzed using binomial generalized linear models (GLM) conducted in R.[34] A *t*-test was conducted in Microsoft Excel to compare the number of hatched nymphs in the first ovarian cycle of females that mated with males fed 0% (control) and 0.5% novaluron.

3 RESULTS AND DISCUSSION

3.1 Population studies

We conducted two-choice and no-choice assays to determine the efficacy of novaluron at different concentrations. Additionally, the two-choice assays provided information on the relative palatability and acceptance of novaluron-supplemented diets, an important consideration for inclusion of novaluron in baits. The two-choice assays showed that *B. germanica* nymphs and adults fed on all novaluron diets, indicating that all concentrations were palatable. The mean number of nymphs in no-choice and two choice assays are shown in Fig. 1 (0% acetone control, 0.0001, 0.001 and 0.01% novaluron) and Fig. 2 (0.025, 0.1 and 0.5%

novaluron). Results for control cages were similar in no-choice assays (0% novaluron acetone control) and two-choice assays (0% novaluron acetone control vs. untreated chow). In these cages, nymphs matured to the adult stage within 15 days and neonates appeared in these cages 50 days after the start of the experiment (Fig. 1A).

The response to 0.0001% novaluron represented a dramatic transition from the response to 0% control treatments to responses to higher doses. In the nymph cages (Fig. 1), almost all the small nymphs died by day 8 in the no-choice and two-choice cages provisioned with 0.0001% novaluron. Some large nymphs survived to the adult stage, but only one produced an ootheca that hatched. At this concentration, there were significant differences in mortality for both small and large nymphs between no-choice and two-choice assays on day 8 (log rank test; small nymphs: $\chi^2 = 11.68$, df = 1, $p = 0.0006$; large nymphs: $\chi^2 = 26.05$, df = 1, $p < 0.0001$). At higher novaluron concentrations (0.001 and 0.01%), however, almost all the nymphs died by day 15 in both no-choice and two-choice assays (Fig. 1C, D). The few nymphs that survived to the adult stage (2% of all nymphs on 0.001% novaluron and 1% of all nymphs on 0.01% novaluron) produced inviable oothecae. At higher concentrations (0.025, 0.1 and 0.5%) all the nymphs died by days 22–29, with small nymphs generally dying within 15 days, and the larger nymphs taking longer (Fig. 2A, B, C). None of these nymphs were able to reach the adult stage and there were no differences between the no-choice and two-choice assays, except for small nymphs at 0.025% novaluron (log rank test, $\chi^2 = 62.4$, df = 1, $p < 0.0001$). Nymphs either died within their exoskeleton or while attempting to molt. These results are consistent with the effect of other BPUs in *B. germanica*, where nymphal mortality was highest during ecdysis and mortality occurred when the emerging nymphs were unable to separate from the exuviae.[5,16]

Adult females in the adults-only control (0% novaluron) cages developed oothecae after 22 days and they hatched within 43 days of the start of the experiment (Fig. 1E). The delayed oviposition and hatching were attributed to the lower temperature of ca. 25 °C in this treatment. The 0.0001% novaluron treatments were conducted at 27 °C and thus, oothecae were observed as early as day 8 and some hatched by day 29 (Fig. 1F). Adult females in the two-choice assays with 0.0001% novaluron were able to produce viable embryos, again demonstrating a 50% dilution of novaluron by untreated chow at this threshold concentration. In the no-choice assays, however, adult females developed oothecae, but all the embryos were inviable and turned black. In all other adult cages provisioned with ≥ 0.001% novaluron in both no-choice and two-choice assays, all females produced only inviable oothecae (Fig. 1G, H; Fig. 2D–F). There was no significant adult female or male mortality in any of the treatment cages, as expected because CSIs do not cause adult mortality in most insects,[1-3] including *B. germanica*.[16]

Overall, these results indicate that novaluron was palatable to nymphs and adults even at relatively high concentrations (0.5%). Although novaluron is not an adulticide, it causes high nymphal and embryo mortality, suggesting that it would be effective in baits for managing German cockroach populations.

3.2 Minimal novaluron intake that interferes with molting

We performed no-choice behavior tests using first instars to determine the minimal duration of exposure to 0.1% novaluron that would interfere with molting. The duration of the first instar is approximately 6–8 days, so we exposed neonates to novaluron for 1, 2 or 3 days. There was

100% survival of nymphs in the control cages (0% novaluron), second instars were observed around day 6 and all first instars molted to second instars by day 8 (Fig. 3). No second instars were found in any of the treatment cages. In four replicate assays of all treatments, 100% of first instars failed to molt and died around days 8–9 (Fig. 3). Overall mortality was significantly higher in all the treatment groups than in the controls (log rank test, $\chi^2 = 391$, df = 3, $p < 0.0001$). Thus, only one day of feeding on 0.1% novaluron (followed by feeding only on untreated chow) was sufficient to disrupt ecdysis and kill all the nymphs.

3.3 Minimal novaluron intake that interferes with reproduction in females

To determine the minimal duration of ingestion of 0.1% novaluron that would disrupt reproduction in females, we performed no-choice tests with newly eclosed females. Females were exposed to 0.1% novaluron for 1, 2 or 3 days before day 5, and then allowed to mate with untreated males. In the control group, females developed oothecae by day 8, retained them for approximately 21 days, and 100% of the embryos successfully hatched between days 34 and 38 (Fig. 4A). In all the treatment groups, females developed oothecae that turned dark brown or black, deformed, and produced no nymphs; nevertheless, females retained these inviable oothecae for approximately 25 days (Fig. 4B). Overall, females were unable to develop viable oothecae and produce nymphs after only one day of feeding on 0.1% novaluron.

These observations are consistent with the ovicidal activity of other BPUs, where the disruption of cuticle formation causes developing embryos to fail to hatch.[1-3] Ovicidal effects have also been shown in adult German cockroaches fed triflumuron,[5,7,8] UC 84572, chlorfluazuron, diflubenzuron,[5] and noviflumuron.[15] These studies determined that the darkened appearance of the ootheca reflected embryonic mortality, which likely occurred during the last two days when the chitinous exoskeleton of the pharate first instar was forming.[5]

German cockroach females carry their ootheca externally during embryonic development, and drop it immediately prior to hatch, or the eggs may hatch while the ootheca is still attached to the female. The anterior end of the ootheca is permeable, which allows transport of water and other materials from the female to the developing eggs.[35] Moreover, the chorion, which envelopes each of the embryos, might also provide for distribution of liquid.[36] This raised the possibility that xenobiotics, including insecticides, ingested by the gravid female might be transferred to the ootheca and affect the embryos.

To assess the effects of novaluron on gravid females, we divided the 21-day gestation period into 5-day intervals and fed cohorts of females 0.1% novaluron chow for 5 days during all phases of gestation. In the control group (no exposure to novaluron), 95% of the oothecae hatched (Table 1). Similarly, in all treatment groups (exposed to novaluron on days 0 to 5, 5 to 10, 10 to 15, and 15 to 20 post oviposition) 95–100% of the oothecae hatched. All the oothecae of females exposed to novaluron for the entire gestation period (days 0 to 20) hatched, showing that novaluron, at this relatively high concentration, does not affect the embryos within the ootheca carried by females.

After they dropped their oothecae, all females were transferred to new Petri dishes and provided a new male. All of the females formed oothecae. In their second ovarian cycle, they were again exposed to the same novaluron treatments as in the first ovarian cycle. All females in the control group (no exposure to novaluron) hatched their oothecae, and in all other treatments (females exposed to 0.1% novaluron on days 0 to 5, 5 to 10, 10 to 15, 15 to 20 or 0 to 20 post oviposition), 90 to 100% of the oothecae hatched (Table 1).

Overall, females exposed to novaluron during oocyte maturation produced inviable embryos, whereas those exposed after the first or second oothecae were formed were unaffected, showing high egg viability and hatch. This reversible sterility was also reported in the Colorado potato beetle, where the majority of eggs produced by females that continuously fed on novaluron-treated foliage failed to hatch, but after a recovery period, females resumed laying viable eggs.[37] Thus, feeding on novaluron during the egg maturation period is necessary to cause ovicidal effects in females. These results suggest that CSIs are delivered to the eggs during vitellogenesis, when German cockroach females feed extensively to provision the eggs and produce the ootheca.[38-40] These observations are consistent with a previous study, showing that females that were fed BPUs during egg maturation in the first ovarian cycle or immediately after the first ootheca hatched produced inviable oothecae.[18]

Experiments with radiolabeled compounds indicated that the anterior escutcheon region of the ootheca is permeable not only to water but also to low-molecular-weight water-soluble materials.[36] Novaluron has relatively low solubility in water and a high molecular weight,[19,31,41] suggesting that it might be transferred to the oocyte bound to hemolymph proteins, but not to eggs already oviposited into an ootheca. Additionally, gravid females feed less compared to other cockroach life stages[39], so there might have been insufficient intake of the active ingredient to affect the embryos.

3.4 Effects of novaluron on males

The effects of novaluron on male mating success and fertility were investigated by treating newly eclosed males, allowing them to mate with normal untreated females and assessing the females' fertility. In all treatments (males fed 0%, 0.1% or 0.5% novaluron chow) 73.3 to 96.7% of the males successfully mated (Table 2). While the effect of novaluron concentration on mating was significant (GLM, $p = 0.026$), there was only a significant difference in mating success between the males fed 0% and 0.5% novaluron (GLM, $z = 2.15$, df = 74, $p = 0.0319$), and no significant differences between the other groups (GLM; 0% and 0.1% novaluron: $z = 1.17$, df = 74, $p = 0.243$; 0.1% and 0.5% novaluron: $z = 1.27$, df = 74, $p = 0.285$). This trend was opposite from expected as significantly more novaluron-treated males mated than control males. Other stimulatory effects have been reported for novaluron, including an increase in fecundity of young adult Colorado potato beetles exposed to novaluron,[42] and greater mass of second instar larvae from eggs treated with novaluron than larvae from control eggs.[43] Hatching rate in the mated females ranged from 93.3 to 100%, indicating that high concentrations of novaluron fed to adult males did not affect their fertility. The effect of novaluron concentration on ootheca hatch was not significant (GLM, $p = 0.295$) and there were no significant differences between any of the groups (GLM; 0% and 0.1% novaluron: $z = 0.279$, df = 63, $p = 0.781$; 0% and 0.5% novaluron: $z = 0.005$, df = 63, $p = 0.996$; 0.1% and 0.5% novaluron: $z = 0.005$, df = 63, $p = 0.996$). Also, there was no significant difference between the number of nymphs that hatched from oothecae resulting from matings with males fed 0% and 0.5% novaluron in chow in the first ovarian cycle (*t*-test, $t = 2.01$, df = 47, $p = 0.171$).

Triflumuron, chlorfluazuron, hexafluron, and UC 84572 were also found not to inhibit male spermatogenesis or spermatophore formation, and were not transferred to females in sufficient amounts during copulation to cause ovicidal effects.[18] However, 0.5% noviflumuron was reported to cause complete sterility in both the first and second ovarian cycles of untreated virgin females that were mated with treated males.[15] This could have occurred through mating, other physical contact, or from horizontal transfer of contaminated male feces to females, as shown for the German cockroach (noviflumuron)[44] and other insect species (diflubenzuron).[45] In our study, males were exposed to novaluron for 13 days, then untreated rodent chow for 24 h before contact with females. This procedure likely minimized transfer of novaluron through non-copulatory routes, such as cuticular contact or in feces.

Since cuticle deposition continues even in the adult stage, we considered that exposure to novaluron might deform or weaken the male genitalia. We examined the phallomere of males that failed to mate; we found no abnormalities, consistent with other reports on the effects of CSIs. Overall, novaluron did not affect mating success and fertility of males and the effects of novaluron were not passed from males to females.

3.5 Effectiveness of novaluron by ingestion vs. topical application

In crop protection, CSIs are most often applied to foliage as residual insecticides. Pest management indoors is more reliant on baits, which are generally safer, more bioavailable, effective, require less AI, and result in less translocation.[46,47] Our comparison of ingestion and topical application demonstrated that both routes of delivery of novaluron are similarly effective in both nymphs and adult females (Fig. 5). In nymphs, 0.1 µg of novaluron was more effective by topical application (62% mortality) than by ingestion (14% mortality) (Fig. 5A), but in females nearly all oothecae were inviable with either mode of delivery of novaluron (Fig. 5B). In both nymphs and adults 100% efficacy was demonstrated by 1 μg of novaluron either by topical application or ingestion.

These results confirm that in our experiments ingestion was the primary mode of novaluron delivery to nymphs and adult females. Although it is possible that cockroaches were also exposed to novaluron through contact, especially in assays that delivered the novaluron-augmented food in small Petri dishes, the dose-response results suggest that most of the novaluron was ingested. Topical application of novaluron was as effective as ingestion, but cockroaches would pick up only a small fraction of the dose by contacting the bait.

3.6 Chitin synthesis inhibitors in German cockroach management

Insect growth regulators that have been registered for German cockroach control are mostly JHAs. Although CSIs have been shown to be effective in managing cockroach populations, they are mainly used to manage crop and forest pests, and to our knowledge have not been commercialized for cockroach control. An experimental gel bait containing noviflumuron was consumed by cockroaches even when alternative food sources (white bread and rodent chow) were present, and more than a fipronil-based cockroach gel bait,[14] showing that baits with CSIs may be competitive with other insecticidal bait products. Novaluron has similar insecticidal activity to noviflumuron and was shown in our studies to be palatable, suggesting that this active ingredient could also be incorporated into cockroach baits. Novaluron in a bait formulation has already shown success controlling subterranean termite infestations,[22] so it is plausible that it might also be effective in managing *B. germanica* infestations.

German cockroaches have developed resistance to active ingredients in some gel bait products, so there is a need to incorporate insecticides with diverse modes of action and formulations into IPM programs to delay and mitigate the evolution of insecticide resistance. Novaluron's unique mode of action, low mammalian toxicity, and efficacy even on IGR-resistant agricultural pests[19-21,48] make novaluron a particularly compelling insecticide for bait development. A primary need is to formulate novaluron into attractive and palatable baits and evaluate its efficacy on field populations of *B. germanica*, likely in combination with adulticides.

Novaluron, like other CSIs, might be effective in preventing the growth of cockroach populations by causing mortality of nymphs and inhibiting adults from producing viable oothecae. Assuming extensive feeding on the bait, suppression of cockroach populations might be achieved faster with CSIs than with JHAs[5,16] because CSIs affect all stages that molt (including embryos) whereas JHAs mainly target the imaginal molt. CSIs also can be used in combination with other active ingredients that serve as adulticides, so that all life stages of the German cockroach are targeted. Moreover, CSIs can be incorporated into bait rotation schemes to manage insecticide resistance.

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CONFLICT OF INTEREST

Parts of this project were supported by Bayer CropScience, and AK is an employee of Bayer CropScience. JH, AW-K and CS have no other conflict to declare.

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Table 1. Percentage of viable embryos within oothecae produced by gravid females exposed to 0.1% novaluron during different five-day intervals and the full 20-day gestation period in the first and second ovarian cycles (*n* = 20 for all treatments).

	First ovarian cycle	**Second ovarian cycle**
Treatment	**% Viable embryos ± SE**	**% Viable embryos ± SE**
Control (no novaluron)	95 ± 4.9	100 ± 0.0
0.1% novaluron days 0–5	100 ± 0.0	100 ± 0.0
0.1% novaluron days 5–10	100 ± 0.0	100 ± 0.0
0.1% novaluron days 10–15	95 ± 4.9	90 ± 6.7
0.1% novaluron days 15–20	100 ± 0.0	95 ± 4.9
0.1% novaluron days 0–20	100 ± 0.0	95 ± 4.9

Table 2. Percentage of successful matings between novaluron-treated males and untreated virgin females and percentage hatch of the resulting oothecae. Within each column, percentages followed by the same letter are not significantly different ($p > 0.05$; GLM).

Novaluron concentration	***n***	**% Mated ± SE**	**% Hatched (of mated) ± SE**
0% (control)	30	73.3 ± 8.1 a	95.5 ± 4.4 a
0.1%	17	88.2 ± 7.8 ab	93.3 ± 6.4 a
0.5%	30	96.7 ± 3.3 b	100 ± 0.0 a

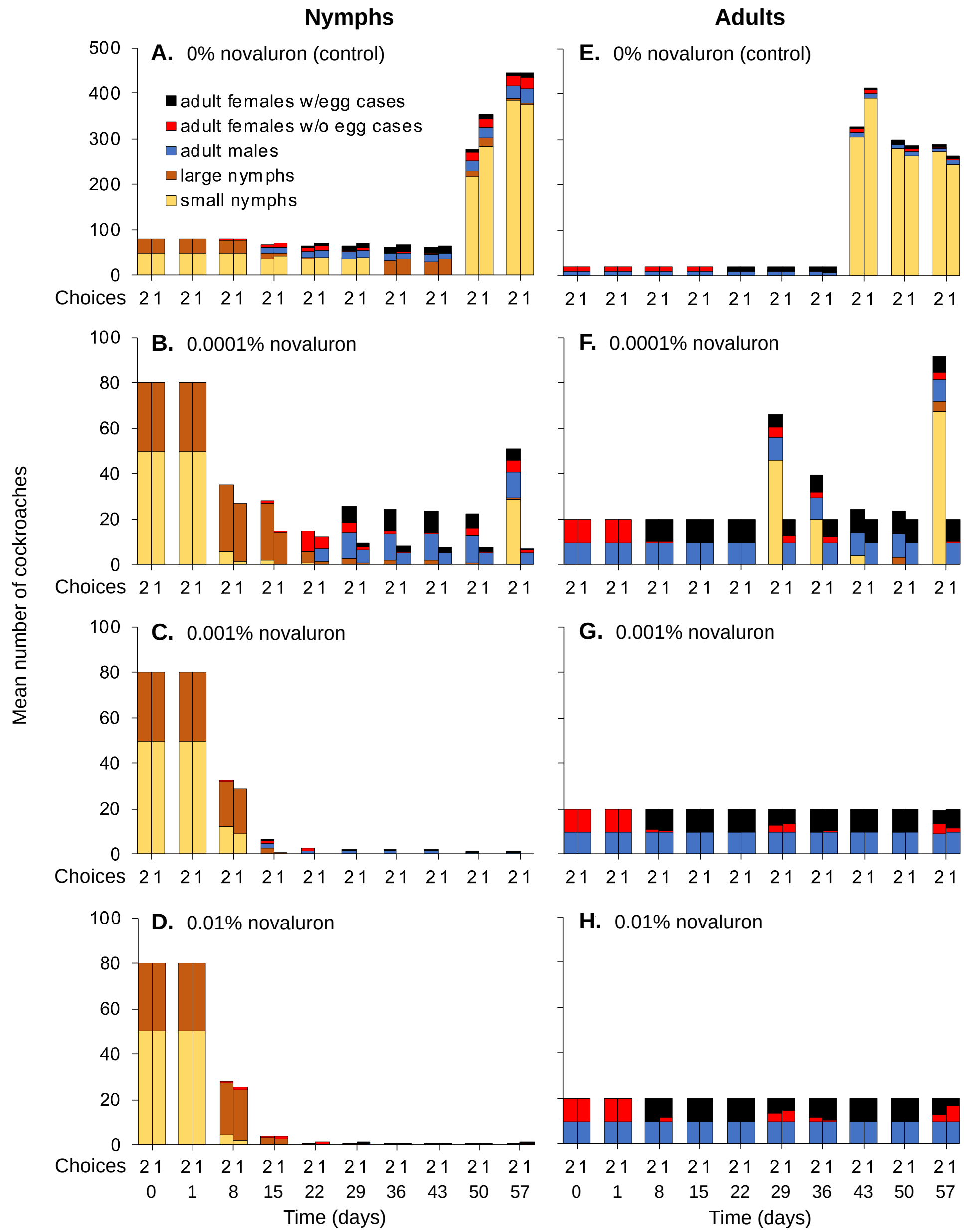

Figure 1. Mean number of cockroaches in two-choice (2 on the X-axes) and no-choice (1 on the X-axes) assays with 0% (control), 0.0001%, 0.001%, and 0.01% novaluron. Each of 3 replicates per treatment started with either nymphs (A–D; 50 early and 30 late instars) or adults (E–H; 10 males and 10 females). Note that the 0.0001, 0.001 and 0.01% novaluron treatments were conducted at 27 °C, whereas the control (0% novaluron) was inadvertently run at a lower temperature of about 25 °C.

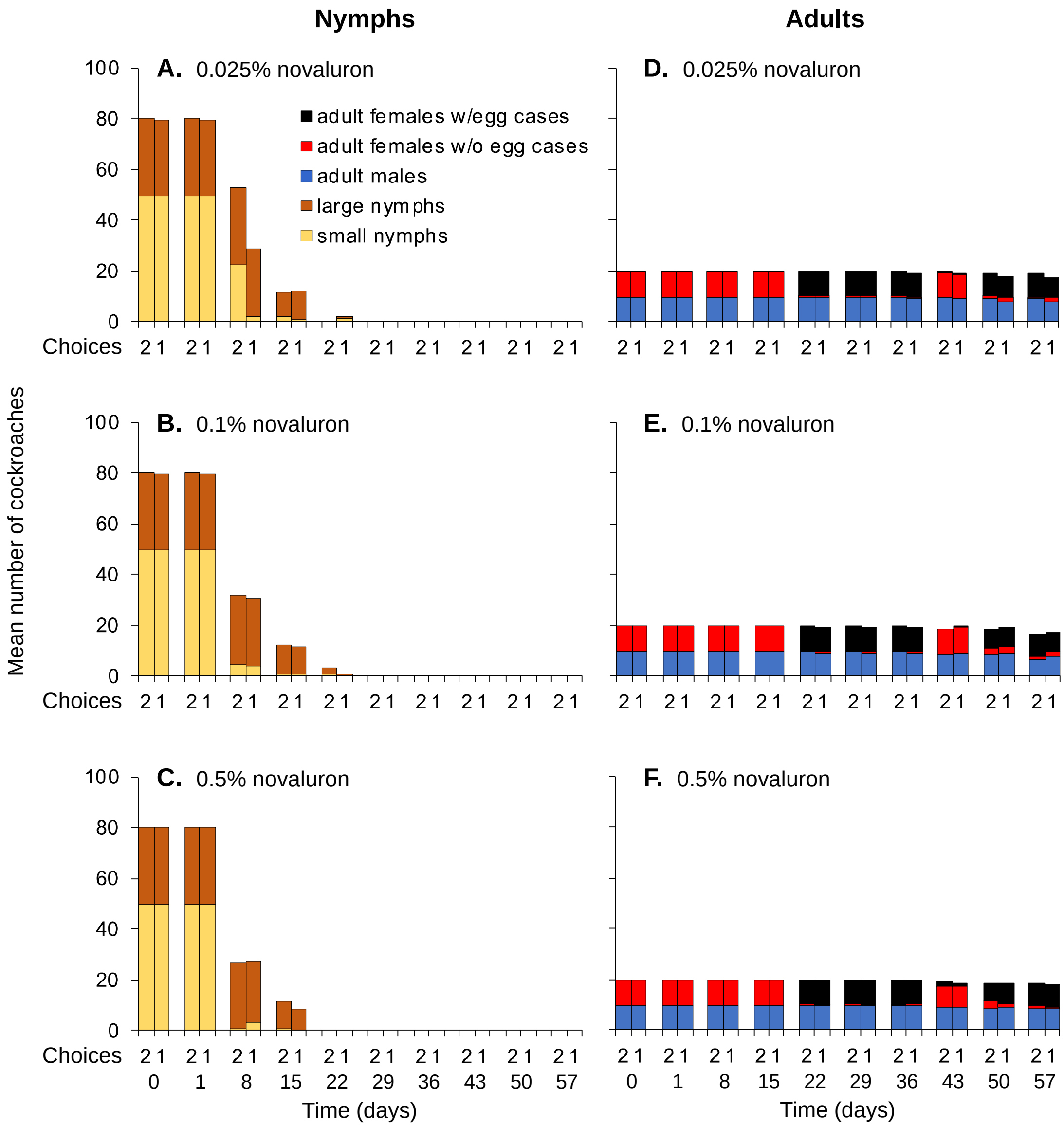

Figure 2. Mean number of cockroaches in two-choice (2 on the X-axes) and no-choice (1 on the X-axes) assays with 0.025%, 0.1% and 0.5% novaluron. Each of 3 replicates per treatment started with either nymphs (A–C; 50 early and 30 late instars) or adults (D–F; 10 males and 10 females). These assays were conducted at about 25 °C.

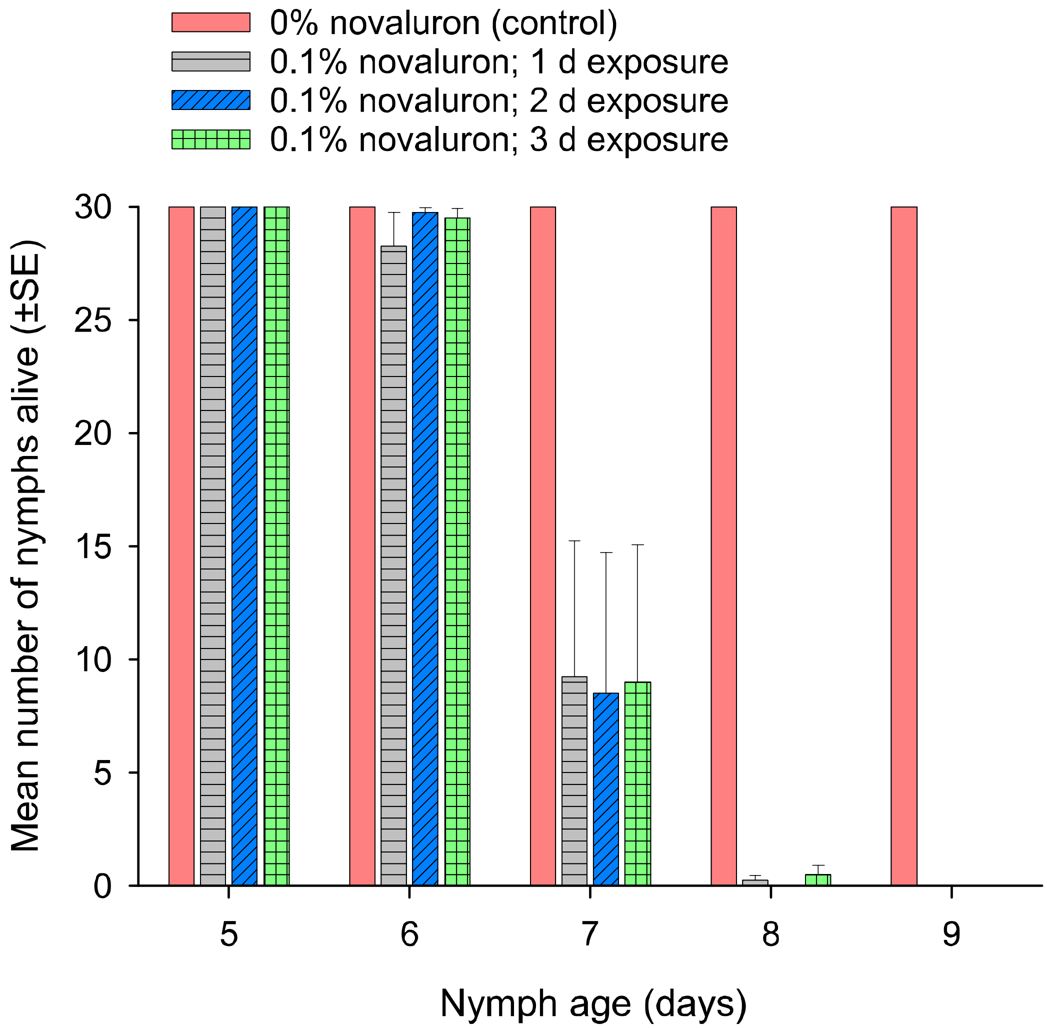

Figure 3. Mean (±SEM) number of nymphs alive on days 5–9 after first instars were fed 0.1% novaluron in rodent chow for 0 (control), 1, 2 or 3 days. There were 4 replicates for each treatment.

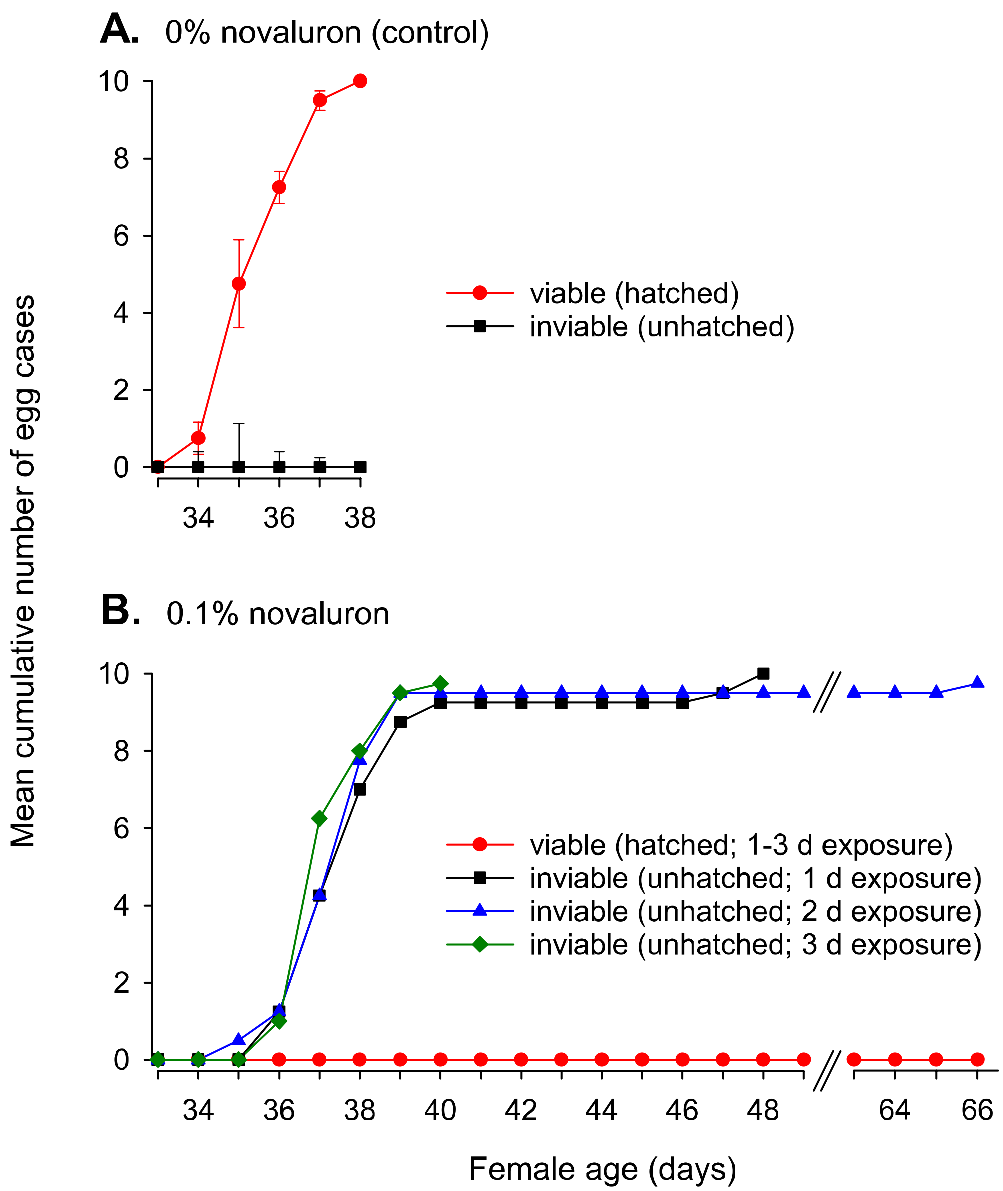

Figure 4. Mean cumulative number of viable and inviable oothecae produced by female cockroaches. Females were either (A) not exposed to novaluron (control), or (B) exposed to 0.1% novaluron for 1, 2 or 3 days. In (A) ±SEM are shown. There were 4 replicates for each group.

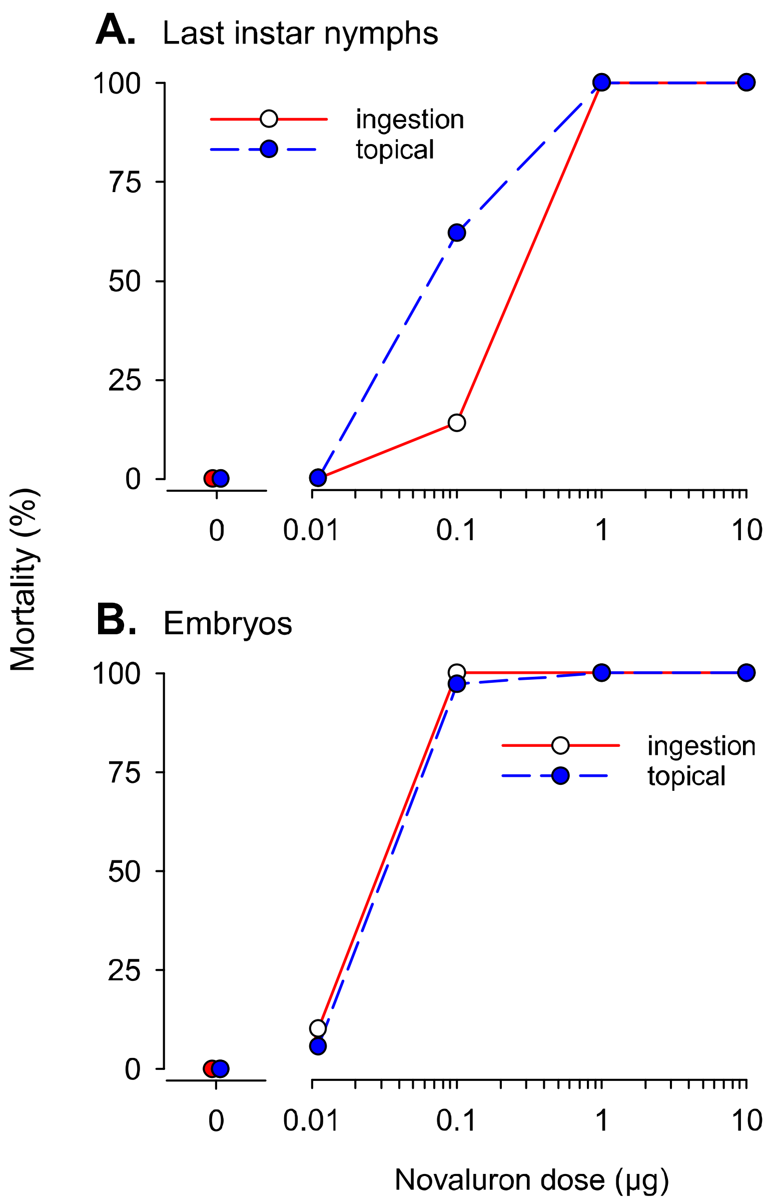

Figure 5. Comparisons of ingested and topically applied novaluron in last instars and adult females. Last instars (A) and adult females (B) were fed 5 mg rodent chow that delivered a known dose of novaluron, or topically treated with a known dose of novaluron in 1 µl acetone (delivered as two successive 0.5 µl applications). Number of cockroaches tested was 21 nymphs and 18–35 adult females per dose.