ABSTRACT

HAYES, CHRISTOPHER CALVIN. The Impacts of Malaria Vector Control Programs on Bed Bugs: History, Insecticide Resistance, and Modern Pressures. (Under the direction of Dr. Coby Schal).

The bed bugs, *Cimex lectularius* L. and *C. hemipterus* (F.), are obligatory hematophagous ectoparasites of the built environment, with infestations arising from small founding populations. Bed bugs preferentially aggregate in tight spaces near the bed and other areas where the potential hosts spend much of their time at night. Bed bugs are largely nocturnal, and typically feed on a sleeping host. Bed bug bites can produce allergic responses, including severe swollen and puss filled lesions and urticaria. They have resurged on a global scale over the past several decades due in large part to the widespread emergence of insecticide resistance. Bed bugs are even more burdensome in malaria-endemic regions.

Malaria, a disease resulting from infection with parasitic protozoans belonging to the *Plasmodium* genus and vectored to humans through the bite of an infected female *Anopheles* mosquito, was responsible for more than 800,000 deaths annually at the turn of the century. Despite decades of progress in malaria control, there are still ~600,000 deaths attributed to malaria annually, making its control a global priority. The most impactful methods of intervention for the past several decades have been the widespread distribution and use of vector control tools, primarily long-lasting insecticide-treated bed nets (LLINs) and indoor residual sprays (IRSs). LLINs are prepared in one of two ways: the historic approach relied on periodically dipping a bed net into a prepared insecticidal mixture, whereas the modern approach incorporates one or more insecticides into the fibers that are used to weave or knit the LLIN. The vast majority of LLINs have used single-ingredient pyrethroid formulations, with dual-ingredient and synergist-containing LLINs only emerging in the past few years. The recommended use of

LLINs requires residents to sleep under them nightly to prevent contact with potentially infected *Anopheles* mosquitoes. However, this approach places LLINs as a potential barrier between bed bugs and their human host.

Herein I have investigated the ecological and behavioral overlap, and interactions of bed bugs with LLINs, first from a historical perspective in the form of a systematic review, and then through several innovative behavioral assays. An examination of the overlap between vector control and indoor pest management shows that the successful ancillary control of indoor pests, including bed bugs, contributed to the adoption of LLINs and IRSs and ultimately to the success of malaria programs. I also document that recent failures to control insecticide-resistant indoor pest populations can lead to the abandonment of LLINs and vector control programs. Thus, there is an immense need to include the efficacy of LLINs on indoor pests in metrics used by the World Health Organization for LLIN approval.

Using robust and innovative behavioral assays with insecticide-susceptible and insecticide-resistant bed bugs, I have shown that commonly deployed single-ingredient and multi-ingredient LLINs do not disrupt host- and aggregation-seeking behaviors and do not repel host-seeking bed bugs. Moreover, bed bugs survive multiple days of continuous contact exposure to these LLINs. Taken together, these results suggest that frequent and sublethal interactions between bed bugs and LLINs likely occur in the field, placing strong selection pressure on already insecticide-resistant bed bug populations. The lack of efficacy of LLINs on bed bugs (as well as other indoor pests), can lead to distrust and abandonment of malaria vector control tools, including LLINs. My findings highlight some of the interactions between indoor pests and vector control tools that likely occur in malaria endemic communities. They underscore the immense

need for further research within this system, particularly under real-world field settings, to support the long-sought goal of global malaria elimination.

The Impacts of Malaria Vector Control Programs on Bed Bugs: History, Insecticide Resistance, and Modern Pressures.

by
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DEDICATION

To my loving wife, best friend, and ever-present advocate Nikki, thank you for your undying support of my academic journey that has spanned nearly a decade of our marriage, and has been filled with peaks and valleys. To my bold, adventurous, and passionate son Atlas, thank you for being a source of constant refinement and joy in my life beyond the lab, and for challenging me to reorient my life to hold graduate school and fatherhood hand-in-hand. To my already energetic daughter Maeva, along with Atlas, I look forward to recounting tales of this journey to both of you as you grow, inspiring passion and purpose within you to accomplish all that you set your mind to. Never stop asking questions, and never stop seeking the answers. To my parents, thank you for the impact you have had on my life from infancy, encouraging my curiosity regardless of our circumstances, and challenging me to always reach new heights. To my sister, thank you for your encouragement and support along this journey, and for all of the journeys we have taken as siblings that have formed me into who I am today. Thank you all.

BIOGRAPHY

Christopher Hayes was born in a largely rural community in western North Carolina. He spent many days of his youth exploring the fields and forests around him, developing in him a love for the natural world around him. He followed this passion and obtained his bachelor's in environmental sciences from North Carolina State University. After graduation Chris pursued a career in the pest management industry, where he quickly learned how critical the control of urban pests is to home- and business-owners and built his foundation in the fields of urban and vector entomology. Chris then obtained his Master of Science in global health from Duke University where he studied malaria vector ecology and distribution in Thailand and Myanmar. This was his first time experiencing the global perspective of pest- and vector-management, and it became an integral experience in the development of his dissertation research. Chris plans to take the skills he has developed over the years and build a career at the intersections of pest management and one health.

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CHAPTER 1

A review on the impacts of indoor vector control on domiciliary pests: good intentions challenged by harsh realities

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Abstract

Arthropod vectored diseases have been a major impediment to societal advancements globally. Strategies to mitigate transmission of these diseases include preventative care (e.g., vaccination), primary treatment, and most notably the suppression of vectors in both indoor and outdoor spaces. The outcomes of indoor vector control (IVC) strategies, such as long-lasting insecticide-treated nets (LLINs) and indoor residual spraying (IRS), are heavily influenced by individual and community-level perceptions and acceptance. These perceptions, and therefore product acceptance, are largely influenced by the successful suppression of non-target nuisance pests such as bed bugs and cockroaches. Adoption and consistent use of LLINs and IRS is responsible for immense reductions in the prevalence and incidence of Malaria. However, recent observations suggest that failed control of indoor pests, leading to product distrust and abandonment, may threaten vector control program success and further derail already slowed progress towards malaria elimination. We review the evidence of the relationship between IVC and nuisance pests and discuss the dearth of research on this relationship. We make the case that the ancillary control of indoor nuisance and public health pests needs to be considered in the development and implementation of new technologies for malaria elimination.

Keywords:

malaria, vector control, malaria elimination, indoor pests, bed bugs, cockroaches

Introduction

(a) The global burden of vector-borne diseases

Vector-borne diseases (VBDs) have directly impacted global populations through high morbidity and mortality, their use as weapons, and more fundamentally by impeding human development and innovation [1]. Examples of these impacts range from the introduction of malaria to susceptible populations during colonization to increasing reports of VBD coinfections [2-4] . Currently, more than 4 out of every 5 people are at risk for VBD transmission, resulting in over one billion cases of VBDs annually, that account for nearly one-sixth of all global illness and disability [5].

The overwhelming burden of VBDs has spurred the advent of control measures following two broad strategies: (1) treatment of known and potential disease hosts to eliminate disease reservoirs, and (2) control of arthropod vectors [6-8]. While these are interdependent strategies, vector control has played a greater role in the global mitigation of VBDs, as it has been the principal (and sometimes only) method of disease management available [9]. Vector control can be divided into two sub-categories, namely outdoors and indoors vector control (IVC). Outdoors or exterior vector control practices focus heavily on vector habitat and breeding site management and disruption, community education and behavior modification, larvicide and adulticide applications, and oviposition site monitoring [10-12]. For the past several decades IVC has relied on the distribution, use, and application of insecticide-based products within homes, primarily targeting *Anopheles* mosquitoes, the vectors of malaria (**Fig 1.1**) [7].

(b) Control of vectors indoors

Historically, IVC measures have included interior applications of insecticides including dichlorodiphenyltrichloroethane (DDT) and more recently pyrethroids, as well as the widespread distribution of insecticide-treated nets (ITNs) and long-lasting insecticide-treated nets (LLINs). These measures have been primarily aimed at disrupting the transmission of malaria, which persists as the most significant vector-borne disease globally [13]. The two bed net types differ in their preparation, with ITNs (historic) being treated by dipping with any World Health Organization (WHO)-recommended insecticide post-manufacturing and requiring frequent retreatment, while LLINs (modern) are manufactured using WHO-recommended insecticide impregnated fibers designed to last up to three years without reimpregnation, making LLINs the most widely adopted indoor intervention today [14]. The use of LLINs and indoor residual sprays (IRS) are integral components of current and historic malaria vector control programs (**Fig 1.1**), with the majority of their deployment occurring throughout Africa, where together they are responsible for over 80% of mitigated malaria cases in Africa from 2000 to 2015 [15-17].

Historically, all WHO-recommended ITNs have been treated exclusively with pyrethroid insecticides (IRAC class 3) [18], modeled after the naturally occurring pyrethrins that target the voltage-gated sodium channel (VGSC) in the insect nervous system and considered relatively safe for human exposure [19]. Pyrethroids, and other newly utilized active ingredients (AIs), including those such as broflanilide, which represent novel modes action aimed at combating resistant vector populations, have replaced DDT and other organochlorine insecticides in IRS applications [20, 21]. Similarly, recent research and funding for LLIN development have gone beyond pyrethroid-only formulations and are focused on combinations of AIs and synergists,

such as co-impregnation with pyrethroids and chlorfenapyr (PermaNet Dual), pyriproxyfen (Royal Guard), and piperonyl butoxide (PBO) (PermaNet 3.0) [22-25]. Despite these recent advances in net design, of the nearly 2 billion distributed treated nets in the past decade, the vast majority are single ingredient pyrethroid-impregnated formulations [26]. Therefore, while efforts to replace nets with dual-ingredient and synergist chemistry are well underway, product development, lag time, supply chain issues, and increased user cost will necessitate the continued reliance on single-ingredient pyrethroid-treated nets for the near future.

(c) Interdependency of vector and pest control

Although the continued programmatic motivation in the development, distribution, application, and evaluation of IVC tools is the reduction of malaria incidence and burden, this is not always the case from the perspective of the user communities. It has been shown for decades that while communities appreciate the long-term health benefits associated with decreased malaria burden, they place as high or higher value on the practical, immediate, and tangible relief from indoor nuisance and biting pests [27]. Indeed, this predilection has been shown by several studies to promote the uptake and adherence to IVC practices by user communities; conversely, when the ancillary benefit of pest control is lost, community perception and adherence to IVC procedures diminishes [28-39]. Yet, a dearth of modern research has investigated the impacts of IVC on indoor pest populations, and subsequently, how domiciliary pest control affects IVC. Most importantly, the risks of abandonment of IVC tactics due to failure to secondarily control domiciliary pests has received little attention. Therefore, we have highlighted the current scarcity of research on this topic and present a case for considering pest management during the design and implementation of the next generation of IVC tools.

(d) Document identification

To identify studies, we conducted a literature review using the Web of Science (WoS) Core Collection database as well as the World Health Organization (WHO) Repository for publications relating to both IVC programs and domiciliary pests (**Fig 1.2**). We searched the relevant literature from 1900 to current publications (29 August 2023) using the following search terms. For the WoS Core Collection: Search 1: ALL FIELDS: (malaria) AND ((bed bug OR bedbug*) OR (cockroach*) OR (filth flies) OR (flea*) OR (nuisance) OR (pest)) OR ((bed bug OR bedbug*) AND (net*)) resulting in 1250 records; and Search 2: TITLE only: (malaria) AND ((indoor residual spray) OR (bed*) OR (net*)) OR (malaria) AND (bednet) OR (bednet*) resulting in 1531 records. For the WHO Repository (1990 – Current): HEALTH TOPIC = "Malaria" OR "Vector Control" OR "Vector-borne diseases" OR "Pesticides" OR "Insecticide treatments" resulting in 527 records. Studies and publications were included if they related to the explicit interaction of domiciliary pests with IVC tools, contained quantified or anecdotal accounts of these interactions, or if they discussed the societal or programmatic implications of these interactions. Non-automated record screening was performed by CCH, with final article selection confirmed by CS, with disagreement on relevancy and inclusion discussed until agreement was reached. While historic bias may exist in regions covered by peer-reviewed material, potentially limiting the scope of available relevant publications, the authors searched without the use of language or journal filters in an attempt to ensure that no relevant articles were excluded from this review.

Initial screening was performed to remove duplicate records, with minimal reliance on automated tools for screening (**Fig. 1.2**). Following initial screening, further assessment of search results was first done based on article topic, followed by title and abstract, with non-

applicable records being dropped. This led to a total of 1,248 records, which were retrieved, and the full text screened for relevance. Full-text screened publications were excluded if they related strictly to agricultural or beneficial nontarget insects, general pest management and biology, general malaria or vector control, or vaccination and human health. This process removed a total of 1,219 records. Additionally, we identified three relevant WHO publications not present in the WHO repository through snowballing of identified publications [41]. We identified a final total of only 28 peer-reviewed publications that met our search criteria, spanning from 1971 to 2022 [28-32, 34-39, 42-53, 83, 85-88], and an additional seven WHO publications spanning from 1968 – 2022 [3, 5, 26, 79-82]. It is important to note that these expansive criteria likely fail to capture historic publications from researchers in frequently underrepresented regions and countries which may be excluded from modern research repositories.

2. An intertwined history: Suppression of domiciliary pests fosters IVC adoption

To understand the association between domiciliary pest control and the success of IVC programs, it is important to discuss their long and codependent history. One of the earliest program evaluations, a blinded survey of nuisance arthropods in Gambian homes, performed in the late 1980s, surveyed 361 homes spanning 6 villages for bed bugs, ticks, and the presence of headlice on children. All participating homes were provided untreated nylon bed nets, and then respective homes received net treatments of diluted cow's milk (placebo = 179) or 500 mg/m^2 permethrin solution (treatment = 182). A four-month post-treatment survey of each home, and each child, revealed nearly double the number of indoor crawling insects in homes provided placebo nets over those using the permethrin-treated bed nets, highlighting the broad impacts and ancillary benefits of net adoption [42]. Lines *et al*. [43] serendipitously reached similar

conclusions when using permethrin-treated bed nets alongside treated curtains to suppress vector populations in Tanzania. Dead cockroaches, lice, and bed bugs were seen throughout study huts and the researchers noted that ancillary pest management would likely appeal to communities more than simply controlling potential mosquito vectors.

Alongside the recorded success of Lines *et al*. [43] in Tanzania, Charlwood *et al*. [44] witnessed similar ancillary benefits. Following the distribution of permethrin impregnated nets for malaria vector control in Papua New Guinea, residents reported decreased bed bug and lice populations. Just two years later, and based on these reports, Charlwood and Dagoro [45] assessed the ability of IVC tools to suppress a major domiciliary pest, the bed bug, by distributing ITNs among workers in Papua New Guinea residing in a bed bug infested bunk house. They found that bed bug populations were indeed heavily suppressed for up to 12 weeks after net distribution, but began to rebound after residents using nets relocated, taking their nets with them. The researchers concluded that not only were the treated nets associated with decreases in bed bug populations, but that this ancillary benefit was the main reason why individuals wanted their nets reimpregnated with insecticide. This work, although small in scope, demonstrated the dual utility of IVC for the control of domiciliary pests.

Follow-up evaluations through the late 1900s and early 2000s demonstrated similar impacts of net adoption on pest populations. In a study of deltamethrin treated curtains, 204 apartment residents were tasked with covering their windows for 1 h at both dawn and dusk and follow-up pest surveys were performed every two weeks for over a year. This intervention resulted in 88–94% reductions in surveyed mosquito vector species and 68–86% reduction in surveyed pest insect species [46]. Similarly, a study aimed to reduce plague incidence in Uganda, evaluated the efficacy of IRS against mosquitoes for controlling flea populations. Five villages

were assigned to each of three groups: normal IRS (wall and ceiling application), modified IRS (wall, ceiling, and a 1 m perimeter floor application), and a no-treatment control. They found that standard IRS application alone resulted in nearly 100% control of flea populations on surveyed rats [47]. These large impacts on domiciliary pests do not go unnoticed by communities, and researchers in Tanzania attempting to quantify community wide ITN impact saw this firsthand. Community members, each provided random nets treated with either permethrin or lambda-cyhalothrin (both pyrethroids insecticides) and surveyed to assess community acceptance and net impact, quickly began approaching members of the study team, "enthusiastically, and without prompting", to inform them of declines in bed bug, flea, and cockroach populations following net deployment. The control of these chronic infestations garnered support for the program within the community, resulted in widespread net reimpregnation and led the researchers to conclude that the main net benefits were reductions in mosquito and bed bug populations [48].

These few studies highlight the impressive ability of IVC tools to secondarily suppress nuisance pests, but it is important to note that none of these studies demonstrated indoor pest elimination. This failure to eliminate pest populations, if left unaddressed, could potentially expose the same populations to strong recurrent selection pressure from AIs (**Fig. 1.1**). Nevertheless, the control of domiciliary pest insects is of high value to communities and represents a major ancillary benefit of IVC program adoption and adherence [49].

3. An intertwined history: Shifting community perceptions underly IVC longevity

Perceptions of ancillary benefits have been shown to shift the motivators for IVC program participation within communities. Indeed, early reports on the perceptions and social factors associated with IVC adoption determined that many communities do so primarily as a reliable

method of domiciliary pest control and only secondarily as a method of disease prevention and vector reduction [50-52]. Temu *et al*. [28] showed a clear example of this in Tanzania, where surveys were performed to assess relationships between bed bug infestations and IVC tool adoption. All surveyed residents from bed bug infested homes, and over one-third of those from homes without bed bug infestations, found bed bugs to be more problematic than mosquitoes. The researchers showed that individuals from bed bug infested homes were not only significantly more likely to purchase and use pyrethroid-treated nets, but also had a 95% reimpregnation rate compared to 77% in uninfested homes [28]. They concluded that successful control of bed bugs (and other domiciliary pests) resulted in higher program adherence, and that these benefits from the use of LLINs should be included in IVC information packages to gain community support.

Years of these collateral benefits have influenced the views of communities and persist today. A recent survey to assess awareness of insect vectors sampled >1000 households of varying socioeconomic backgrounds and education levels from all 10 districts in Botswana. They found that 58.9% of respondents were most concerned with indoor mosquitoes, but nearly 40% were most concerned with cockroaches and house flies [53]. It is easy to see how these two major and differing perceptions could arise in the same communities, and they hold the power to facilitate or stall IVC program rollout and adoption. This is not surprising, as the adoption and success of any vector control program is influenced not only by biological, climatic, and socio-economic factors, but also by human perceptions [54]. The potential short transition from programmatic support to rejection in the wake of pest resurgence is not uncommon. While perceptions of IVC success are clearly rooted in lower vector and disease prevalence, often overlooked is the unintended suppression of nontarget pests that can drive residents to enthusiastically adopt and maintain IRS and LLIN regimes (**Fig. 1.1**). Failing to recognize and

value the sway of domiciliary pest control on community perceptions can impede IVC programs even after measurable success is realized in their early adoption.

4. IVC programs can select for insecticide resistance in domiciliary pests

The independent evolution of insecticide resistance in various populations of disease vectors is a major barrier to vector control programs [55]. Likewise, heavy selection pressure through the overuse or misuse of insecticides favors insecticide resistance alleles in indoor pest populations. Two of the starkest examples of this are in the German cockroach (*Blattella germanica*) and the bed bug (*Cimex lectularius* and *C. hemipterus*).

The German cockroach, arguably the most impactful indoor pest on public health, burdens resource-constrained communities globally and its distribution is tied closely to the movements and behaviors of humans [56]. The introduction of German cockroaches is often accidental, or a result of spill-over in multi-unit or adjoining homes, and infestations can quickly reach thousands in the absence of effective control measures. Severe infestations result in large deposits of frass (shed exoskeletons, pulverized dead cockroaches and fecal matter), that contain high levels of allergens [57, 58]. Prolonged exposure to these allergens has been shown to impact pulmonary health by contributing to the development of asthma [57, 59]. Additionally, German cockroaches have been shown to harbor a diverse gut and fecal microbiome, implicating them in the transmission of pathogens under certain circumstances [58, 60]. These burdens are exacerbated in low-income and resource-limited communities, especially when control measures are confounded by insecticide-resistant cockroach populations, despite the ability to mitigate these burdens with targeted, low-cost, and proven control measures [59, 61].

The bed bug, a persistent domiciliary pest that has resurged globally to unprecedented numbers in the past two decades, also places an immense burden on individuals and communities on nearly every continent [62-65]. Small founding propagules can quickly erupt into major infestations in the absence of effective eradication measures [66]. Although bed bugs are largely considered a nuisance pest, the bites of this obligatorily hematophagous insect are associated with allergic responses ranging from mild itching and swelling to inflamed and painful rashes, to pus-filled bullous lesions, and in rare cases, anemia [67]. Further, bed bug feces contain high levels of histamine, an immune modulator associated with itching, swelling, and even anaphylaxis [68]. Because of these varying but potentially severe repercussions associated with bed bug infestations, their elimination from homes is a global priority.

The German cockroach and bed bug are highly adapted to human-built structures. They do not fly, have high-fidelity for recognized aggregation sites, and are rarely found in the outdoor environment (e.g., bed bugs associated with bats) (**Fig 1.1**). As such, populations are isolated by the same artificial barriers that delimit our homes, with minimal gene flow between populations. Thus, adaptive alleles that confer resistance to insecticides quickly become fixed in these populations. In the case of the German cockroach, varying levels of resistance to 42 AIs have been shown dating back to the 1950s [69-72]. Similarly, varying levels of resistance to multiple classes of insecticides have been found in bed bug populations, including organochlorines, organophosphates, carbamates, pyrethroids, neonicotinoids, and pyrroles [73]. Resistance has even been recorded to the phenylpyrazole class of insecticides in bed bugs, namely to the AI fipronil, despite no products containing fipronil being labeled for the control of bed bugs [74]. These examples highlight the remarkable propensity of these two pervasive indoor pests to rapidly evolve adaptive resistance mechanisms following the introduction of

novel chemistries, and to maintain that resistance long after those insecticides are phased out of use.

The impact of IVC on domiciliary pests is not limited to German cockroaches and bed bugs, and it was widely documented across a variety of indoor pests in the 1960s by Busvine and Pal [75]. A WHO-designed survey was sent to more than 100 health authorities worldwide, and more than half responded. The surveys collectively indicated widespread insecticide resistance in many non-target species from regions with ongoing IVC, including in house flies and fleas. House fly populations across the world have evolved resistance to most products used in their control [76]. More relevant to VBDs, high resistance to a variety of insecticides has been observed in many flea populations, including the cat flea (*Ctenocephalides felis*), human flea (*Pulex irritans*), and likely as a result of DDT-based malaria efforts in the rat flea (*Xenopsylla cheopis*) (>5000-fold resistance) [77, 78]. Flies and fleas pose direct risks to human health, often as co-infestations with other pests (**Fig. 1.1**), and such widespread resistance is at least in part a result of failing to consider the impact of IVC on indoor pests.

The WHO is aware of the impact of IVC tools on domiciliary pest populations, and the resulting effect of indoor pests on vector control programs. Indeed, a 1968 WHO publication [79] recognized that insecticide resistance typically emerges in indoor pest populations several years following application of IVC products. There-in the WHO underscored that resistance in bed bug populations can lead to community interference with IVC application, as up to 80% of participants refused IRS reapplication. The solutions proposed included surveys, education programs, and insecticide treatments separate from malaria programs. While each of the proposed strategies may have merit as part of an integrated bed bug control program, it was not well integrated with IVC efforts [79]. In addition, the WHO evaluation and recommendations

revolved around a single pest, despite the potential of other indoor pests to disrupt IVC. Recent WHO publications concerned with insecticide resistance and best use practices for IVC tools are nearly exclusively focused on the mitigation of insecticide resistant vector populations [80-82], which is a critical component of IVC success. We posit, however, that decades of negligence in managing insecticide resistant indoor pest populations in malaria endemic areas, and the miniscule current body of research on the continued impacts of IVC on pest populations, will equally hinder future IVC product development, rollout, and success.

5. A dearth of contemporary research

We have summarized the interconnected history of IVC and domiciliary pests, the community expectation of IVC tools to control indoor pests, and the propensity of obligate and opportunistic domiciliary pests to rapidly evolve insecticide resistance mechanisms when exposed to IVC measures. Yet, few contemporary researchers have investigated how well modern IVC tools control indoor pests. The lack of updated information jeopardizes the efficacy of IVC programs, as many of these tools are used in conjunction with one another, overlapping with areas in the home frequented by nontarget pests (**Fig. 1.1**).

Bed bugs, a principal example of this overlap, are the major focus of the minimal modern research that exists on IVC and indoor pest ecology. Due to their need for a blood meal every few days, bed bugs rest in relative proximity to humans throughout their lives. A study performed in Magugu Tanzania assessed pyrethroid resistance in bed bugs (*C. lectularius*) through exposure to net swatches and pyrethroid treated filter papers. Tested AIs included varying concentrations of deltamethrin, permethrin, alpha-cypermethrin, and lambda-cypermethrin [83]. Of 1500 adult bed bugs assayed, ~50% were from homes without LLINs, but

unfortunately the results were not stratified by LLIN use. The 24 hr LD_{95} was between 3.3X (permethrin) and 15X (alpha-cypermethrin) higher than the estimated LD_{50}. A reference susceptible strain was not included so resistance ratios could not be estimated, but extrapolating from other reports [84], these populations might have been highly resistant to permethrin. Similarly, the most recent LLIN bioefficacy study, performed in Cape Coast, Ghana, explicitly assessed LLIN efficacy for bed bug control. In a survey of 205 homes, 66.3% were found to be infested with bed bugs (*C. hemipterus*). Collected bed bugs were exposed to deltamethrin- or alpha-cypermethrin-impregnated LLINs for 72 hr, and unsurprisingly only 4% and 12% mortality was seen, respectively [85]. Additional bed bugs from homes with low-level treatment were also assessed, but only 44% to 86% mortality was obtained. These studies present a case for pyrethroid resistance in bed bugs resulting in widespread failure of IVC tactics (including IRS and LLINs) to suppress bed bug populations.

Studies from other regions have demonstrated similar concerning trends following the use of LLINs. For example, Myamba and colleagues [86] investigated the resurgence of populations of *C. hemipterus* in villages in the Tanga region of Tanzania which had ongoing LLIN use for six years, with annual retreatment. Bed bugs collected from homes in five villages that adopted LLINs and five without LLINs, and their resistance to pyrethroid-treated bed nets and filter papers was assessed using contact assays. Shockingly, they saw only 4–51% mortality in bed bugs from villages with prolonged LLIN use, up to 72 hr after a continuous 1 hr exposure. In contrast, all but a single group of the bugs collected from control villages experienced >92% mortality. Similarly, researchers in India found 100% mortality in vector species after 3 min of LLIN exposure, but >30 min was needed for 100% mortality of house flies and American cockroaches (*Periplaneta americana*), and only 25% of bed bugs collected from local homes

died after 30 min of exposure [87]. A critical consideration not addressed in these reports and rarely mentioned in related studies is how long indoor pests remain in contact with LLINs or IRS in residential settings (**Fig. 1.1**). The requirement of 30 to 60 min of continuous exposure to obtain 100% mortality is potentially unrealistic and fails to consider the complexities of pest behavior and interactions with IVC tools.

Only one recent study addressed the behavioral interactions of an indoor pest with LLINs. It assessed the ability of various bed bug (*C. lectularius*) life stages, fed and unfed, of both insecticide susceptible and resistant strains to pass through several LLINs in pursuit of a blood meal or aggregation [88]. Most bed bugs regardless of life stage or feeding status were unhindered in their passage through two LLINs with differing weave and AIs, Olyset (permethrin) and PermaNet 2.0 (deltamethrin). Additionally, <30% mortality was found after 1 hr potential exposure time in an insecticide susceptible strain during feeding assays and <65% mortality after 24–168 hr potential exposure in aggregation assays. None of the pyrethroid resistant bed bugs died in any of these assays. While it remains unclear whether bed bugs in the field engage in brief passages through the bed net in pursuit of blood meals and harborage, or rest on the LLIN for extended periods of time, these results suggest the former. Notably, the biology of bed bugs dictates recurrent interactions with LLINs, which likely have been occurring for decades in malaria-endemic regions, and have exerted intense selection pressure on populations, driving the emergence of insecticide resistance and leading to anecdotal observations of bed bugs aggregating directly on LLINs.

Interactions of other hematophagous pests, such as fleas (*Ctenocephalides* and *Xenopsylla* spp.), with LLINs likely follow similar dynamics to bed bugs. However, in these holometabolous insects only adults are exposed to the LLIN selection pressures. Other household

pests, such as the German cockroach and filth flies, are not expected to have frequent interactions with LLINs, and are likely affected more by IRS (**Fig. 1.1**). Nevertheless, the behavioral interactions of indoor pests with various IVC tools needs to be characterized, the repellency of IVC products and their AIs to indoor pests and resistant strains needs to be evaluated, and the distribution and frequency of resistant genotypes in indoor pest populations across malaria control program sites needs to be quantified.

6. Intensifying selection pressures: Adding fuel to the fire

Clear gaps in our understanding of the interactions of domiciliary pests with currently recommended IVC tools remain, yet the next generation of products are poised to dramatically intensify selection pressures, especially on bed bugs. First, novel LLIN designs incorporate synergists (inhibitors of detoxification enzymes, such as PBO) and multiple AIs to overcome insecticide resistant vector populations. Indeed, the addition of PBO to LLINs increased mortality in highly pyrethroid resistant mosquitoes in Uganda; however, bioefficacy of the bed nets declined quickly over the course of two years due to dissipation of the synergist [89]. Despite this reported success, the addition of PBO to LLINs is not expected to provide substantial benefits against highly resistant mosquitoes. Similarly, varying success of the addition of PBO has been shown against highly resistant bed bug strains [74, 82, 90], which for decades have continued to develop extremely high levels of resistance to pyrethroids [85, 91].

Another developing strategy to combat resistant vector populations is to incorporate new chemistry into both LLINs and IRS. For example, the combined use in Benin of alpha-cypermethrin (pyrethroid) treated LLINs (DuraNet) with each of three non-pyrethroid IRS (bendiocarb [carbamate], chlorfenapyr [pyrrole], and fipronil [phenylpyrazole]) significantly

increased mortality in pyrethroid resistant mosquitoes [92]. Varying levels of resistance to some of these potential products, including to the aforementioned insecticides, has already been seen in domiciliary pest populations [55, 61, 77, 93-98]. Once again, the design and assessment of novel vector control strategies fails to consider the status of domiciliary pest populations, and how these products may confound control of these populations by further contributing to insecticide resistance. Therefore, we predict that indoor pest populations, namely bed bugs, will adapt to new LLIN and IRS faster than mosquito vectors due to already high resistance, persistent interactions with IVC at all life stages and low gene flow between 'closed' sedentary populations. This rapid adaptation, together with insecticide resistant vectors, increase the risks of net abandonment, individuals preferentially resting outdoors to escape homes inundated with biting pests, and a growing distrust of IVC products, including LLINs and IRS [99].

7. Concluding remarks: A pressing need

We have discussed the critical roles of indoor pest control and community perception for the early success and continued acceptance of IVC programs and tools. In recent years, however, the fight against malaria has stalled and there is evidence of increasing malaria incidence in many communities[26, 100]. The contribution of the unabated surge of insecticide resistant domiciliary pests (**Fig. 1.1**) to the distrust and abandonment of IVC tools is unknown. The failure of emerging IVC technologies to consider their impacts on these pests dates back to the introduction of DDT and persists due to limited AI formulations. This dearth of AIs with differing modes of action, and the long-term deployment of LLINs resulting in the delayed rollout of new products, place severe constraints on resistance management in the fight against malaria. There are no immediate solutions to this challenge. However, as a first step we urge

greater consideration of several key principles in the design, testing and implementation of current and proposed IVC tools:

a. IVC products should be rigorously evaluated for their ability to simultaneously suppress vector and domiciliary pest populations.
b. Greater emphasis should be placed on the discovery and development of inorganic AIs with physical or mechanical modes of action, such as silica and boric acid, which effectively control even highly insecticide resistant bed bugs [101, 102].
c. The frequencies, levels, and distribution of insecticide resistance in major indoor pests should be monitored in concert with monitoring of vector populations.
d. The prevalence and burden of nuisance pests (filth flies), biting insects (bed bugs, fleas, lice), and obligatory indoor pests (bed bugs, German cockroach) should be assessed as part of IVC rollout and reevaluation (**Fig. 1.1**).
e. The changing perspectives and concerns of malaria endemic communities regarding IVC effectiveness should be repeatedly surveyed to ensure lasting adherence to new and established IVC technologies.

While this is not an exhaustive list, nor one that guarantees the success of IVC moving forward, it represents several crucial steps in our collective efforts to revitalize the stalled progress in the fight against malaria. IVC must take a more integrated approach, focusing not only on vector control but simultaneously on indoor health and wellbeing, including indoor pest management, an approach that will more effectively support the goal of malaria elimination.

Abbreviations

AI	Active Ingredient
DDT	Dichlorodiphenyltrichloroethane
IRS	Indoor residual spraying
ITNs	Insecticide treated nets
IVC	Indoor vector control
LD	Lethal dose
LLINs	Long-lasting insecticide-treated nets
PBO	Piperonyl butoxide
VBDs	Vector-borne diseases
WHO	World Health Organization

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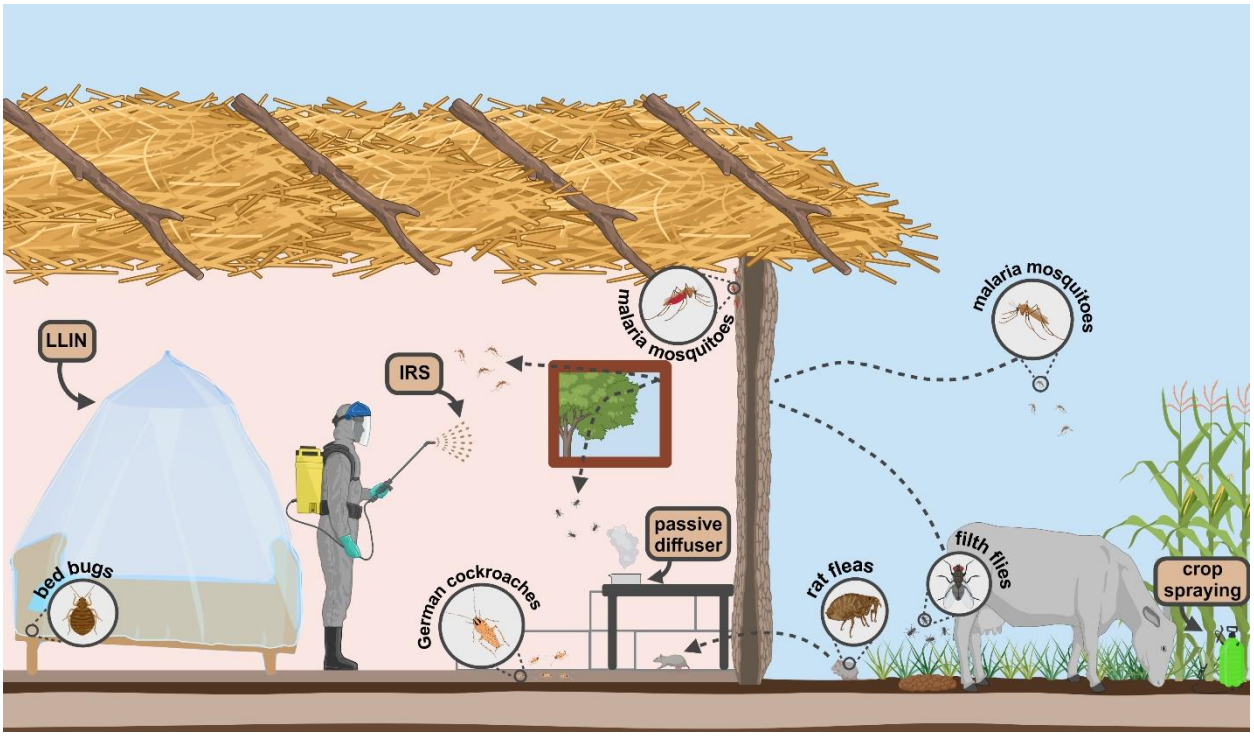

Figure 1.1. The overlap of indoor vector control tactics and domiciliary pests. The success of indoor vector control in malaria-endemic communities can be impacted by obligate domiciliary pests and transient pests that occasionally enter the home. Outside the home, highly mobile insects (house fly, *Musca domestica*; flea, *Ctenocephalides* and *Xenopsylla* spp.) are exposed to insecticides outdoors via targeted or crop sprays. In the home, obligate indoor pests with low mobility (bed bug, *Cimex* spp.; German cockroach, *Blattella germanica*) and those that have entered the home from outside are exposed to insecticides from consumer products (e.g., passive insecticide diffusers, aerosols), indoor residual sprays (IRS), and insecticide treated bed nets (ITNs), including long-lasting insecticide treated nets (LLINs). Each of these nontarget exposures exerts strong selection pressure on indoor pests in malaria endemic communities, leading to the widespread emergence of insecticide resistant pest populations, which greatly hinder and disrupt vector control programs. Figure created with BioRender.com.

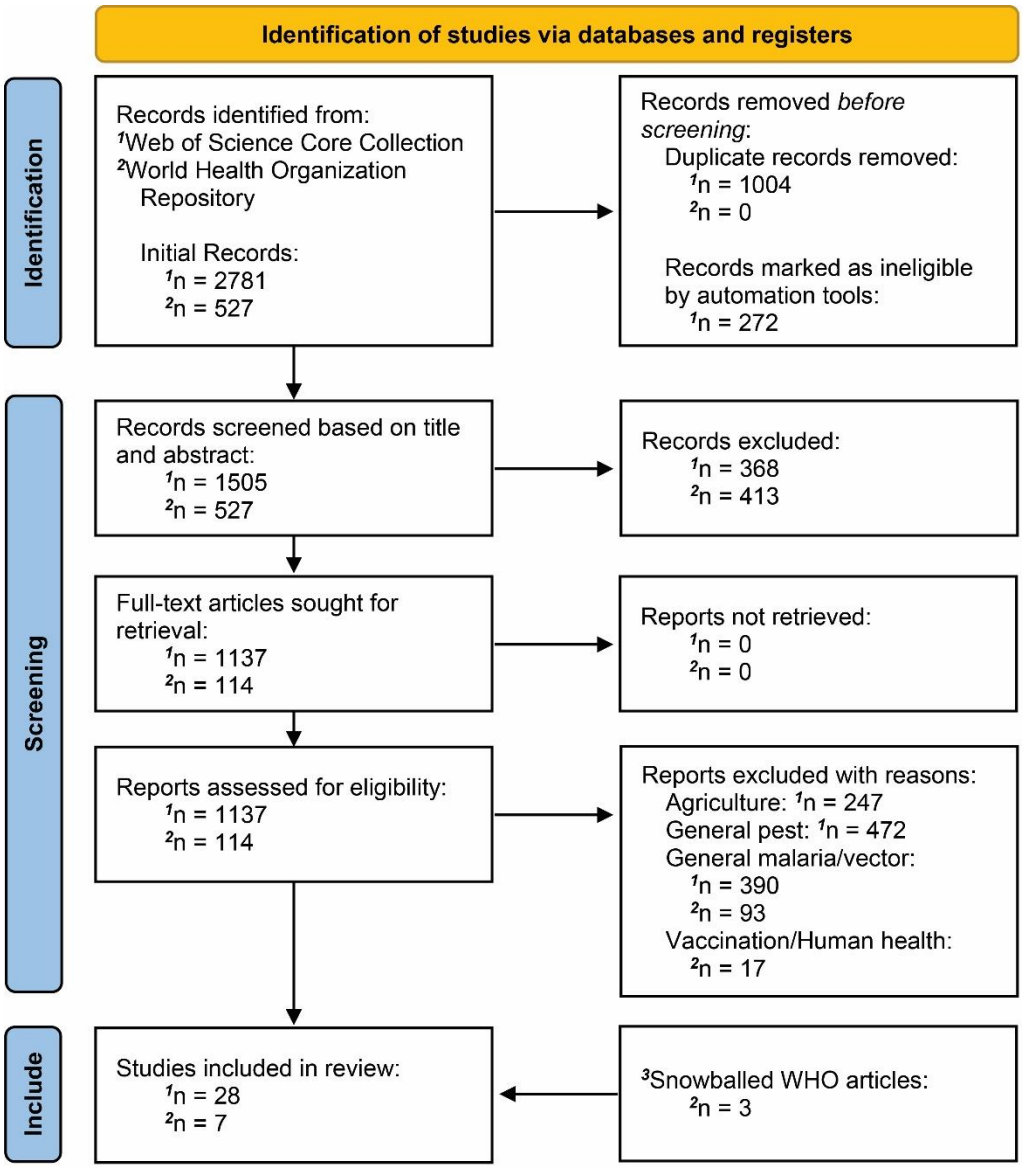

Figure 1.2. Flowchart of the selection process for articles related to the interaction of domiciliary pests with IVC tools. Comprehensive searches were performed following the PRISMA guidelines as closely as possible (see http://www.prisma-statement.org/) [40] across two repositories: Web of Science Core Collection and the World Health Organization Publication Repository. Searches covered publications spanning from 1900 to August 2023.
[1]In reference to records obtained from the Web of Science Core Collection Database.
[2]In reference to records obtained from the World Health Organization Publication Repository.
[3] Snowballing: "Reviewing the citations of systematically identified relevant publications for additional material."[41].

CHAPTER 2

Behavioral interactions of bed bugs with long-lasting pyrethroid-treated bed nets: challenges for vector control

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Abstract

Background

Widespread vector control has been essential in reducing the global incidence and prevalence of malaria, despite now stalled progress. Long lasting insecticide-treated nets (LLINs) have historically been, and remain, one of the most commonly used vector control tools in the campaign against malaria. LLINs are effective only with proper use, adherence, retention and community adoption, which historically have relied on the successful control of secondary pests, including bed bugs. The emergence of pyrethroid resistant bed bugs in malaria endemic communities and failure to control infestations have been suggested to interfere with the effective use of LLINs. Therefore, the behavioral interactions of bed bugs with commonly used bed nets should be better understood.

Methods

To investigate the interactions between bed bugs (*Cimex lectularius* L.) and LLINs, insecticide-susceptible and pyrethroid-resistant bed bugs were challenged to pass through two commonly used LLINs in two behavioral assays, namely host (blood meal)-seeking and aggregation-seeking assays. The proportions blood-fed and aggregated bed bugs, aggregation time and mortality were quantified and analyzed in different bed bug life stages.

Results

Overall, both the insecticide-susceptible bed bugs and highly resistant bed bugs showed a varying ability to pass through LLINs based on treatment status and net design. Deltamethrin-treated nets significantly impeded both feeding and aggregation by the susceptible bed bugs. While none of the tested LLINs significantly impeded feeding (passage of unfed bed bugs through the nets) of the pyrethroid resistant bed bugs, the untreated bed net, which has small

mesh holes, impeded passage of fed bed bugs. Mortality was only seen in the susceptible bed bugs, with significantly higher mortality on deltamethrin-treated nets (63.5 ± 10.7%) than on permethrin-treated nets (2.0 ± 0.9%).

Conclusions

Commonly used new LLINs failed to prevent the passage of susceptible and pyrethroid-resistant bed bugs in host-seeking and aggregation-seeking bioassays. The overall low and variable mortality observed in susceptible bed bugs during both assays highlighted the potential of LLINs to impose strong selection pressure for the evolution of pyrethroid resistance. Already, the failure to control bed bug infestations has been implicated as a contributing factor to the abandonment or misuse of LLINs. For the first time, we have shown the potential of LLINs in selecting for resistant secondary pest populations and so their potential role in stalling malaria control programs should be further investigated.

Keywords: *Cimex lectularius*, Malaria, Long-lasting insecticide nets, Resistance evolution, Insect behavior

Introduction

Malaria and the role of bed nets in malaria control

Vector borne diseases (VBD), those diseases that are transmitted through the bite of an infected arthropod vector, threaten nearly 82% of the global population and disproportionately affect those living in the poorest areas [1]. Malaria, a parasitic protozoan disease vectored to humans through the bite of an infected *Anopheles* mosquito, is the most widely studied VBD and remains one of the most severe public health problems worldwide [2]. Efforts to reduce the prevalence and incidence of malaria have historically been relatively successful [3]. Despite these advances, however, recent research has shown that malaria remains endemic in all World Health Organization (WHO) regions, and that substantial previous gains in malaria control have slowed [4]. Today, nearly half the world's population lives in malaria-endemic areas. Accurate surveillance of disease incidence and malaria control presents a major challenge in some countries while others are moving closer to malaria elimination [5].

Mosquito control has been indispensable in reducing both the incidence and prevalence of malaria globally, being alone responsible for a roughly 33% decrease in malaria burden across Africa from 2000 to 2010 [6-8]. Commonly used malaria vector control practices include environmental management to remove oviposition sites, larvicide use, the application of residual insecticides to interior surfaces of homes known as indoor residual sprays (IRS), area-wide spraying, the use of novel baits and the widespread deployment of pyrethroid-treated bed nets known as long-lasting insecticide-treated nets (LLINs) [9, 10]. LLINs are the most widely distributed and used method of vector control due to their effectiveness at both repelling and killing mosquitoes indoors and thus reducing biting rates, low cost, sustainability and reliability to reduce the burden of malaria transmission across a varied epidemiological landscape [11].

Adherence to bed net use and their retention, both of which rely heavily on community perception and commitment, are key to effective vector control, reducing biting rates and the successful reduction and elimination of malaria from endemic regions.

Impact of secondary pests on vector control

Homes in low- and low-middle income countries, where vector control measures are commonly deployed indoors, are often infested with other hematophagous pests, such as kissing bugs, fleas, lice, and most notably, bed bugs. Unlike mosquitoes, all mobile life stages of bed bugs are hematophagous and flightless, and therefore they live in close proximity to humans. Whereas *Cimex lectularius* is primarily distributed in temperate regions, *Cimex hemipterus* is found primarily in tropical regions [12, 13]; however, populations of both species overlap and have been shown to co-infest dwellings in communities across Africa, Asia, Australia, North America and more recently Europe, mainly through global travel [14-18]. Propagules of just a few bed bugs can quickly expand into large infestations in the absence of effective control measures, due to their tolerance of inbreeding, relatively high fecundity, rapid development, long adult life and cryptic nature [19]. Bed bugs are generally considered a nuisance pest, as they are not known to vector any pathogens to their human hosts. However, their bites have the potential to cause severe allergic reactions, painful inflammation, itching, as well as anemia in rare cases associated with severe infestation [20]. Additionally, bed bug feces contains large amounts of histamine, a multi-functional immune modulator and neurotransmitter known to cause muscle swelling, itching and other allergic responses, further highlighting the potential harmful effects associated with bed bug infestations [21]. Therefore, effective control of bed bug infestations is a priority for residents globally, regardless of income status.

Many communities that use LLINs prefer to focus on "practical or secular concerns" such as the control of indoor urban pests, rather than the long-term national and generational health benefits associated with decreased disease transmission and vector control [22]. Thus, in resource-limited malaria-endemic communities, where bed bug control is unaffordable or unavailable, LLINs are heavily relied upon for bed bug control [23]. As a result, the control of bed bugs can directly impact bed net uptake and adherence to their proper use [22]. This effect was shown in the late 1990s in Tanzanian communities where, for example, >50% of sampled homes reported bed bug infestations, and the perceived suppression of bed bugs by LLINs led to significantly greater net uptake and retreatment rates, as well as greater adherence to bed net use [24].

By the early 2000s, however, anti-malaria campaigns suggested that dichlorodiphenyltrichloroethane (DDT)-resistant bed bugs impeded malaria control efforts through disruption of community adherence, leading to refusal of DDT treatments [25]. Even now, communities in Sub-Saharan Africa continue to rate the effectiveness of distributed LLINs on their ability to kill secondary pests around their homes, including house flies, fleas, cockroaches and bed bugs; low efficacy of LLINs on secondary pests can result in net abandonment and misuse [26-29]. Additionally, widespread bed bug infestations in Rwandan communities despite LLIN interventions were a "major hindrance" to the continued effective use of these vector control tools [30]. These studies highlight the intricate connection between LLIN use for vector control in malaria endemic communities and collateral effects, namely the impact of LLINs on secondary indoor pests. Emerging evidence suggests that inefficacy of LLINs in the control of secondary indoor pests, especially bed bugs, can significantly disrupt LLIN adoption and their sustained use.

Insecticide resistance in bed bugs may disrupt LLIN use

Insecticide resistance is a major impediment to timely suppression and eradication of bed bug populations [31]. Bed bugs have a long history of evolving and maintaining resistance to insecticides, with the first major example being resistance to DDT observed in 1947, which persists in populations today [32, 33]. Modern examples include resistance to other organochlorine insecticides, carbamates, organophosphates, pyrethroids, neonicotinoids and fipronil (phenylpyrazole; not labeled for the control of bed bugs) [33, 34]. These patterns demonstrate that bed bugs rapidly evolve adaptive responses to insecticides that target them. Moreover, the unintentional exposure of bed bugs to insecticides not labeled for their control may impose strong selection pressure and lead to the evolution of insecticide resistance. LLINs may similarly expose bed bugs to pyrethroid insecticides. Indeed, recent research has shown that exposure of bed bugs to common LLINs in the field resulted in low mortality even in homes where no prior chemical control specifically targeted against bed bug was reported [23].

Selection by LLINs for pyrethroid resistance in bed bug populations may disrupt LLIN-based malaria control efforts. Behavioral interactions of bed bugs with LLINs are therefore the likely setting where selection for pyrethroid resistance occurs. To our knowledge, however, there are no studies of bed bug-bed net interactions. Therefore, we designed behavioral assays that required host-seeking and aggregation-seeking bed bugs (*C. lectularius*) to interact with and pass through pyrethroid-treated bed nets and insecticide-free bed nets. It is imperative to discern what role, if any, LLINs may play in influencing or disrupting these two behaviors. Understanding these interactions may mitigate barriers to LLIN use and guide the development and implementation of more effective bed nets to achieve malaria elimination while also suppressing bed bug infestations.

Methods

Colony maintenance and feeding

Two laboratory-maintained strains of *C. lectularius* were used in this study. The Harlan strain (also known as Ft. Dix) is an insecticide-susceptible strain commonly used as a reference. It was collected at Fort Dix, New Jersey (USA) in 1973 and has not been challenged with insecticides since collection. The Harlan strain was maintained on a human host until December 2008, then on defibrinated rabbit blood until July 2021 and on human blood thereafter. The Fuller Mill strain was collected in a residence in High Point, North Carolina (USA) in 2017. Compared to the Harlan strain, the Fuller Mill strain has ~1,000-fold resistance to permethrin (Hayes and Schal, unpublished) and 44.4-fold resistance to fipronil [34]. Both bed bug colonies were maintained at 35–45% relative humidity, 25 ºC on a photoperiod of 12:12 (light:dark) h, and fed weekly on heparinized human blood (supplied by the American Red Cross under IRB #00000288 and protocol #2018-026). We used an artificial feeding system, which has been previously described [35]. The feeding system was housed in a North Carolina State University-approved BSL-2 facility (Biological Use Authorization # 2020-09-836). Between feeding sessions, the glass feeders were sanitized with 7.5% sodium hypochlorite and 95% ethanol, and air-dried.

Bioassay apparatus design

We designed and validated two bioassays that assessed the passage of bed bugs through various bed nets. The first assay, the blood meal-seeking assay, quantified the passage of unfed, host-seeking bed bugs through bed nets. This assay was designed on the premise that unfed bed bugs are highly motivated to seek a blood meal and are highly responsive to host-emitted stimuli [36].

The second assay, the aggregation-seeking assay, quantified the passage of fully fed, refuge-seeking bed bugs through the net. This assay was based on evidence that fully fed bed bugs rest near the host but are deterred from arresting (resting) on host-produced lipids [37]. Both bioassays used a two-jar system with a net "sandwiched" between the two jars (**Fig. 2.1**). The "net sandwich" was prepared by hollowing the solid lids of two plastic jars (5.5 cm x 4.8 cm each; Olcott Plastics, Saint Charles, Illinois (USA)), securing a section of bed net between them with glue (Gorilla Glue, The Gorilla Glue Company, Cincinnati, Ohio (USA)), and compressing it until it dried.

The edges of the bed net cutting were trimmed and the joint sealed with stretched parafilm. The solid bottoms of both jars were replaced with tightly woven plankton netting (BioQuip Products, Rancho Dominguez, California (USA)) chemically bonded with dichloromethane. The top netting provided all life stages access to the blood, and both the top and bottom netting provided air circulation through the bioassay apparatus. Each of the two jars also contained a section of file folder (Manila) paper that ensured firm contact with the bed net and the plankton net (**Fig. 2.2**). These papers provided a walkway that enabled unfed bed bugs to access and cross the bed net to feed and fed bed bugs to cross the bed net to rest in a dark aggregation site. After each bioassay, all jars were gently washed by hand with warm water and dish detergent.

Bed net and bed bug dimensions

New bed nets were provided by the U.S. Centers for Disease Control (CDC) and were selected as representative samples of untreated and commonly used and distributed insecticide-treated nets. The bed nets were 1) Siam Dutch, an untreated bed net (Siam Dutch Mosquito Netting Co.,

Bangkok, Thailand); 2) Olyset Net (Sumitomo Chemical, Osaka, Japan), a permethrin-treated LLIN; and 3) PermaNet 2.0 (Vestergaard, Lausanne, Switzerland), a deltamethrin-treated LLIN (**Table 2.1**).

We empirically measured the length and width of 20 individual mesh holes in each bed net using an ocular micrometer in a microscope (Zeiss Stemi 47 50 57, Zeiss Microscopy, Jena, Germany). Additionally, we measured the width of the widest thoracic segment and widest point of the abdomen, in both unfed and fully fed bed bugs. We measured 20 representative bed bugs of each life stage of both the Harlan and Fuller Mill strains using the same microscope used for the bed net measurements.

Blood meal-seeking assay

Unfed bed bugs were placed in the bottom jar and challenged to cross the bed net to obtain a blood meal. Six to seven adult females were placed in the top jar of each assay to stimulate activation behavior. In each assay we used either a mixed stage cohort of 50 bed bugs consisting of 10 each of 2^{nd}, 3^{rd}, and 5^{th} instars, and adult males and females (n = 5 replicates), or a single cohort of 40 2^{nd} instars (n = 5 replicates). All bed bugs were 10–14 d post feeding, which ensured that they were highly motivated to feed. Bed bugs were held in a 5.5 cm x 4.8 cm jar for up to 20 min in the dark at 20–40% relative humidity and 25–26°C, while the feeding apparatus was prepared. Each feeding membrane was gently dabbed three to four times on the forearm to transfer human body odor to the membrane. The jar holding the bed bugs was then attached to the bed net-sandwich assembly and three to four breaths were exhaled into each top jar to activate bed bugs with CO_2 and human odors.

Each assay system was placed on a feeder for 1 h. All assays were conducted during the scotophase (night time), in a darkened room, at 20–40% relative humidity and 25–26ºC. The total number of blood-fed insects was recorded, representing the number of bed bugs that passed through the bed net. It is important to note that this assay did not account for bed bugs that might have crossed the net but failed to feed. Therefore, the assay recorded the minimum number of bed bugs that crossed the net. We also recorded the number of moribund (knocked down but able to respond to stimuli) and dead bed bugs (those no longer showing any response to stimuli) by gently touching bed bugs with forceps.

Aggregation-seeking assay

In this assay, fresh fully fed bed bugs were placed in the top jar of the bioassay assembly and challenged to cross the bed net to reach a dark resting site. Newly molted 2nd instar bed bugs were starved 7–10 d and fed for 1 h in a 5.5 cm x 4.8 cm jar at 20–40% relative humidity and 25–26ºC without exposure to a bed net. Only fully fed bed bugs were selected and placed in a new jar that contained a fresh file folder paper walkway. This jar was connected as the top jar to the bed net-sandwich assembly. The bottom jar served as the target for aggregation-seeking fed bed bugs. To make this jar attractive to fed bed bugs, we "conditioned" file folder paper by exposing it to a colony of bed bugs, which impregnated it over time with feces and aggregation pheromone. A section of the aggregation-pheromone impregnated paper was placed within the target (bottom) jar, and the jar was wrapped completely in aluminum foil to create an attractive darkened aggregation site [38]. Further, the whole bioassay assembly was placed horizontally in a 25ºC incubator on a photoperiod of 12:12 light:dark h at 35–45% relative humidity. The combination of the attractive paper and darkness served to attract bed bugs toward the

aggregation jar, while the incubator lights repelled bed bugs away from the exposed starting jar. We counted and removed bed bugs from the target aggregation jar after 1 h, and then every 24 h, during the photophase (day time), for 7 d. At each check, we removed the aggregation jar from the assay assembly, counted and removed the bed bugs (including dead bugs) and reconnected the aggregation jar to the bioassay system.

Statistical analysis

All statistical analyses were conducted using SAS Enterprise Guide (v. 8.3, SAS Institute, Cary, NC), with $\alpha = 0.05$. We compared the hole lengths (longer dimension) and widths (shorter dimension) of the three bed nets with separate one-way ANOVAs. The pre- and post-feeding thoracic and abdominal widths of bed bugs from both strains were compared using two-tailed paired Student's *t*-tests. The thorax and abdomen widths of bed bugs (different life stages, different strains, unfed and fed) were compared to the mesh hole length of each bed net using one-way ANOVA followed by Dunnett's multiple comparison tests, setting the bed net as the control to which bed bug sizes were compared. Results of the blood meal-seeking assays (proportion of bed bugs that traversed the net and blood-fed) were arcsine-square-root-transformed and compared between the two strains and across the three bed nets using a 2-way ANOVA. The results of the aggregation assays (proportion of bed bugs that traversed the net and aggregated in the dark jar over time) were arcsine-square-root-transformed and compared using a 2-way ANOVA. Mean time to aggregation was determined across bed net-strain pairs using survival analysis, and cumulative mortality on the bed nets was compared with the non-parametric Wilcoxon signed-rank test.

All reported data were confirmed to be normally distributed, and measures of variation around the mean are standard errors (SE). Where box plots are shown, each represents the interquartile range (IQR, the difference between the third quartile and the first quartile), median, minimum and maximum values if there are no outliers or 1.5 times the inter-quartile range if outliers are present (represented by whiskers), outliers (if present), and mean (represented by x).

Results

Bed net and bed bug dimensions

The three bed nets we used differed in their insecticide treatment and the size of mesh holes through which bed bugs would need to pass. The untreated bed net (Siam Dutch) had relatively symmetrical holes measuring 1.39 ± 0.05 mm by 1.38 ± 0.13 mm (n = 20). The deltamethrin-treated bed net (PermaNet) was similar in hole dimensions to the untreated bed net (1.65 ± 0.04 mm by 1.33 ± 0.07 mm, n = 20). The permethrin-treated bed net (Olyset) had much larger oblong holes, measuring 3.78 ± 0.25 mm by 1.95 ± 0.24 mm (n = 20) (**Fig. 2.3**). Analysis of the nets revealed that hole lengths were significantly different across the three bed nets (F = 1333.07, df = 2, 57, P < 0.0001) (**Fig. 2.3**). Only the Olyset net had significantly wider holes than the other two nets (F = 102.91, df = 2, 57, P < 0.0001). Therefore, based on hole dimensions alone, we expected the bed nets with smaller holes to selectively allow only smaller bed bugs to pass through, whereas bed bugs should be least constrained passing through the Olyset net.

We used a total of nearly 3,800 bed bugs in the assay validation and data collection phases of this project. We measured the maximal thoracic and abdominal widths of various developmental stages of unfed and fed bed bugs of both strains (**Fig. 2.4**). Overall, the maximal thoracic and abdominal widths of fed bed bugs were similar to the respective widths of unfed bed

bugs. In some stages (e.g., 2nd instars, adults), fed bed bugs were significantly narrower than unfed bed bugs, indicating that the enormous increase in body mass associated with full engorgement on a blood meal resulted in longitudinal stretching of the abdomen and an increase in its girth that stretched the pleural membrane, decreasing the width of the abdomen. The mean thorax width of Harlan strain bed bugs ranged from 0.49 ± 0.01 mm in fed 2nd instars to 2.12 ± 0.03 mm in fed adult females (n = 20 for each stage) (**Fig. 2.4a**), and in Fuller Mill bed bugs from 0.80 ± 0.02 mm in unfed 2nd instars to 1.97 ± 0.03 mm in fed adult females (n = 20 for each stage) (**Fig. 2.4b**). Mean abdominal widths in Harlan bed bugs ranged from 0.88 ± 0.03 mm in fed 2nd instars to 3.24 ± 0.03 mm in unfed adult females, and in Fuller Mill bed bugs from 1.06 ± 0.03 mm in unfed 2nd instars to 2.86 ± 0.03 mm in unfed adult females.

In general, the Harlan and Fuller Mill bed bugs broadly overlapped in their thoracic and abdominal widths. The thoracic widths of fed 2nd instars and adult male Harlan bed bugs were significantly smaller post feeding (Student's t-tests, t = 13.16, df =38, $P < 0.001$; t = 2.39, df = 38, P = 0.0219). Harlan 2nd instars, as well as adult males and females had significantly smaller abdominal widths after feeding (Student's t-tests, t = 3.10, df = 38, P = 0.0042; t = 5.46, df = 38, $P < 0.001$; t = 5.17, df = 38, $P < 0.001$). Interestingly, the width of the thorax in Fuller Mill bed bugs did not change significantly in any life stage, whereas abdominal widths were significantly smaller in fed than unfed adult males and adult females (Student's t-tests, t = 5.11, df = 38, $P < 0.001$; t = 6.36, df = 38, $P < 0.001$); the reverse was evident in 2nd instars, with larger abdominal widths in fed nymphs (Student's t-test, t = 2.50, df = 38, P = 0.018).

To estimate which bed bug stages might be excluded by each of the three bed nets, we statistically compared the maximum widths of each stage of unfed and fed bed bugs, respectively, with the hole lengths of each bed net. Resulting significant differences in size were

associated with either a predicted ability to pass through the respective net (smaller size), or with a predicted exclusion by the net (larger size), independently of the effects of insecticides. For brevity, only those life stages around the "cut-off" point, where exclusion or passage may be based on feeding status, as determined by statistical comparison, are presented. In the case of the untreated (Siam Dutch) bed net, we found that only 2nd instars of both Harlan and Fuller Mill were significantly smaller than the measured hole length ($F = 596.51$, $df = 40, 779$, $P < 0.0001$; Dunnett's test, $P < 0.05$), and therefore both fed and unfed 2nd instars were predicted to pass through this net. Interestingly, most 3rd instars of both strains were predicted to pass through unfed (Dunnett's test, $P < 0.05$), but might potentially be excluded once fully fed (Dunnett's test, $P > 0.05$) (**Fig. 2.4**). Similar results were seen with the deltamethrin-treated (PermaNet) net, as 2nd instars of both strains, as well as 3rd instar Harlan bed bugs were expected to pass regardless of feeding status ($F = 606.38$, $df = 40, 779$, $P < 0.0001$; Dunnett's tests for both, $P < 0.05$). However, most 3rd instar Fuller Mill bed bugs, as well as most 5th instars of both strains were predicted to be excluded once fully fed (all 3 Dunnett's tests, $P > 0.05$) (**Fig. 2.4**). In the case of the permethrin-treated (Olyset) net, all measured life stages of both strains were significantly smaller than the mesh hole length, and so most were predicted to pass through the net regardless of feeding status ($F = 674.45$, $df = 40, 779$, $P < 0.0001$; Dunnett's tests for all, $P < 0.05$) (**Fig. 2.4**).

Blood meal-seeking assays with multi-stage bed bug cohorts

To empirically investigate the predictions of statistical tests, we assayed mixed cohorts of Harlan strain bed bugs of all previously measured life stages to identify which would pass through the nets and which would not. We compared the untreated Siam Dutch bed net, with small mesh

holes and no interference from insecticides, and the permethrin-treated Olyset bed net, with large mesh holes. In the blood meal-seeking assays, 2nd, 3rd, and 5th instars readily passed through the untreated bed net, with mean feeding percentages of 100%, 98%, and 94%, respectively, whereas 82.7%, 86%, and 96% passed through the permethrin-treated bed net and blood-fed (**Fig. 2.5**). Due to the large variation among replicates with the permethrin-treated bed net, we found no significant differences in the responses of 2nd, 3rd, and 5th instars on the untreated and permethrin-treated bed nets ($P > 0.05$), which generally aligns with our statistical analysis of net hole length and bed bug size. Of note, however, is that while the passage of 2nd and 3rd instar unfed bed bugs through the untreated net was expected based on our prior analysis, the passage of unfed 5th instars was not. This potentially relates to the pliable nature of the nets, which in certain cases might allow bed bugs to pass through successfully, as well as the more pliable nature of the bed bug abdomen than the thorax.

On the other hand, the untreated net completely excluded adult males and females from reaching the blood source (0% fed), whereas on average 69.6% and 63.9% of the males and females, respectively, traversed the permethrin-treated bed net and fed (**Fig. 2.5**). The 2-way ANOVA analysis showed a significant effect of the overall model on bed bug blood feeding. Bed bug life stage, LLIN used, and the interaction between stage and net significantly influenced the proportion of successfully blood-fed insects (**Table 2.2**). Specifically, the passage and feeding of adult bed bugs through the two nets were significantly different (**Fig. 2.5**) and aligned with the predicted results from our prior analysis. These results suggest that while bed nets with tight mesh (small holes) may stretch, and bed bugs may alter the width of their abdomen, these adjustments are constrained, as all adults were prevented from traversing the untreated net. Conversely, and again in line with our prior analysis, the permethrin-treated bed net, with large

holes, permitted all life stages to pass through, suggesting that the insecticide did not repel or deter unfed, host-seeking bed bugs.

Blood meal-seeking assays with 2nd instars of two bed bug strains

Because 100% of 2nd instars of the Harlan strain passed through the untreated control bed net (**Fig. 2.5**), we compared their responses to those of pyrethroid resistant bed bugs (Fuller Mill) on all three bed nets. Overall, in all replicates with both strains, ≥65% of the nymphs traversed the three bed nets and blood fed (**Fig. 2.6**). The 2-way ANOVA analysis showed a significant effect of the model on successful blood feeding. All factors assessed, bed bug strain, bed net and the interaction of strain and net significantly impacted blood feeding (**Table 2.2**). Throughout the assay Harlan strain bed bugs appeared to be more active than Fuller Mill bed bugs (CCH, personal observation), and a higher percentage of Harlan 2nd instars (96.5%) than Fuller Mill nymphs (85.5%) passed through the untreated bed net and fed (Tukey's HSD, $P = 0.0006$) (**Fig. 2.6**). The responses of both strains were similar on the permethrin-treated Olyset bed nets, and in line with our prior analysis of net hole length and unfed bed bug width, with 94.0% of Harlan bugs and 90.0% of Fuller Mill bugs feeding (Tukey's HSD, $P > 0.05$). Compared to the untreated bed net, lower percentages of 2nd instars of both strains passed through the deltamethrin-treated PermaNet bed nets and fed (Harlan: 72.0%, Tukey's HSD, $P < 0.0001$; Fuller Mill: 79.0%, $P = 0.0009$) (**Fig. 2.6**). These findings contrast with the predictions based on our comparison of net hole length and bed bug width, suggesting that deltamethrin may impede passage through the PermaNet bed net. In support of this, we found no significant differences in passage through the untreated net and the permethrin-treated Olyset bed net (Tukey's HSD, $P = 0.2428$).

There was no mortality of Harlan or Fuller Mill bed bugs in the 60 min assay with untreated nets. However, 27% and 29% of the Harlan 2nd instars were moribund after the 60 min assays on permethrin- and deltamethrin-treated bed nets, respectively. In contrast, none of the pyrethroid-resistant Fuller Mill bed bugs experienced any morbidity.

Aggregation-seeking assays

The aggregation-seeking assay was designed to simulate the passage of freshly fed bed bugs through a bed net in search of a refuge or aggregation site. We hypothesized that fed and unfed bed bugs would differ in their maneuverability through the three bed nets. This 7-day assay assessed bed bugs as they transitioned from a fully fed state to an unfed state. Across all three bed nets, and throughout the duration of the assay, all fully fed Harlan bed bugs crossed the untreated and permethrin-treated bed nets and 100% were found in the aggregation jar within 24 h; <80% crossed the deltamethrin-treated net during the 7-day assay (**Fig. 2.7a**). Some Fuller Mill bed bugs failed to cross all three bed nets even after 7 days and thus <100% were found in the aggregation jar by the end of the assay (**Fig. 2.7b**).

From survival analysis, we estimated the mean aggregation times (time to 50% aggregation) for the Harlan bed bugs as 1.6 ± 0.25, 1.6 ± 0.25, and 14.8 ± 1.38 h through the untreated, permethrin-treated, and deltamethrin-treated bed nets, respectively (**Fig. 2.7a**). Correspondingly, mean aggregation times for the Fuller Mill nymphs were 33.6 ± 2.84, 5.0 ± 0.71, and 12.3 ± 1.16 h through the same bed nets (**Fig. 2.7b**). Cumulative aggregation for both strains across all three nets was compared at 1 h, 24 h, and 168 h via 2-way ANOVA. The model revealed a significant effect of the interaction between bed net and strain only at 1 h, and significant effects for both the net and strain predictor variables, as well as the interaction

between bed net and strain were seen at 24 and 168 h (**Table 2.3**). Overall, there was no significant effect of the permethrin-treated bed net on movement of the Harlan or Fuller Mill nymphs at any of the three time points (Tukey's HSD tests, $P = 0.18$, $P = 1.00$, and $P = 1.00$, respectively), but a significant effect of the deltamethrin-treated bed net throughout (Tukey's HSD, $P = 0.0001$ for all 3 time points).

Across all three nets the final cumulative aggregation of fed 2nd instar bed bugs ranged from 47.5% (PermaNet) to 100% (Siam Dutch, Olyset) in Harlan, and from 52.5% (Siam Dutch) to 98.5% (Olyset) in Fuller Mill (**Fig. 2.7c**). The 2-way ANOVA of aggregation at 168 h revealed a significant effect of the overall model on bed bug aggregation ($F = 10.64$, $df = 5, 24$, $P < 0.001$). In fact, bed bug strain, LLIN used and the interaction of strain and LLIN all significantly impacted 168 h aggregation (**Table 2.3**). As expected from our prior comparison of net hole length and bed bug width, we found no significant effect of the untreated or permethrin-treated bed net on the 168 h aggregation of Harlan bed bugs (Tukey's HSD, $P = 1.00$) (**Fig. 2.7c**). However, significantly fewer Harlan nymphs had traversed the deltamethrin-treated net by 168 h (Tukey's HSD, $P = 0.0001$), once again suggesting the potential role of a barrier beyond hole size, likely the presence of deltamethrin. In line with our prior statistical comparisons yet again, we found no significant effect of the permethrin- or deltamethrin-treated bed nets on aggregation of Fuller Mill bed bugs at 168 h (Tukey's HSD, $P = 1.00$), but unexpectedly, significantly fewer Fuller Mill nymphs traversed the untreated bed net throughout the assay compared to the permethrin-treated bed net (Tukey's HSD, $P = 0.0032$) (**Fig. 2.7**).

Movement through the untreated bed net toward the aggregation jar caused no mortality in bed bugs of either strain during the 7-day assay (**Fig. 2.8**). However, interactions of fully fed Harlan strain nymphs with the permethrin- and deltamethrin-treated bed nets resulted in 2.0 ±

0.9% and 63.5 ± 10.7% mortality, respectively. There was no mortality in the resistant Fuller Mill bed bugs on these two bed nets. Thus, there was no significant difference in mortality of the Harlan and Fuller Mill bed bugs when interacting with the permethrin-treated bed net (Wilcoxon signed-rank test, $Z = 1.81$, $df = 1$, $P = 0.1667$). However, the Harlan nymphs experienced significantly higher mortality than the Fuller Mill bed bugs on deltamethrin-treated bed nets (Wilcoxon signed-rank test, $Z = 2.67$, $df = 1$, $P = 0.0079$) (**Fig. 2.8**).

Discussion

To our knowledge, this is the first study to quantify the passage of bed bugs through commonly used LLINs in two distinct contexts, host-seeking by unfed bed bugs and shelter-seeking by fed bed bugs. Through the use of innovative behavioral assays, we were able to show that the presence of commercial grade LLINs impregnated with permethrin or deltamethrin had no effect on these two critical bed bug behaviors in a highly resistant strain, and only low, but varying effects on a highly susceptible strain which is unlikely to be seen in the field. Thus, our findings suggest that complete bed bug control is unlikely to be an ancillary benefit of current LLIN use. Instead, a large proportion of unfed bed bugs would be expected to readily pass through these bed nets to obtain blood meals from sleeping human hosts. Likewise, a large proportion of fully fed bed bugs would readily pass through bed nets returning to shelter and aggregation sites. Only some of the highly insecticide-susceptible bed bugs succumbed to the LLINs, and no pyrethroid-resistant bed bugs died.

We suspected that passage of bed bugs through bed nets would be limited by the mesh (hole) sizes of various nets and repellency associated with the presence of pyrethroid insecticides. As expected, physical interactions of bed bugs with bed nets, namely the

dimensions of net holes in relation to bed bug size, affected which life stages of bed bugs could pass through each bed net to obtain a blood meal or return to harborage. Surprisingly however, pyrethroid-impregnated bed nets provided marginal additional barriers, beyond the physical barriers, to prevent the passage of bed bugs through bed nets. Overall, our findings suggest that the high motivation of unfed bed bugs to orient toward a blood-meal host, and high propensity of fed bed bugs to follow aggregation stimuli, likely contribute to recurrent lethal and sublethal interactions with LLINs, which in turn would select for the evolution of pyrethroid resistance in bed bug populations.

Interactions of bed bugs and humans with LLINs

Bed bugs tend to aggregate on or near the bed or other furniture where humans rest and sleep, although at high population densities they may be more broadly distributed and shelter farther away from the bed. In general, bed bugs do not linger on the host beyond the several minutes required to take a blood meal, in part repelled or deterred by triglycerides associated with human skin [37]. Unfortunately, the behavioral ecology of the association of bed bugs with LLINs has not been previously investigated. Additionally, human behavior is expected to influence the interaction of bed bugs with bed nets. Correct usage of bed nets requires that the mattress be fully enclosed within the bed net each night [39]. This practice would likely enclose some bed bugs that aggregate on the mattress within the net, thus minimizing their passage through the LLIN, but would serve as a potential barrier to other bed bugs on the bed frame and around the home. Therefore, these bed bugs would then need to pass through the bed net at least twice every 7–14 days throughout the life of each bed bug to obtain at least one blood meal before each of its five molting events and to support the maturation of each batch of eggs and sperm [36,40].

Our results suggest that when LLINs are properly used, bed bugs may need to alter their behaviors to obtain a blood meal. For example, more tightly woven nets, such as the PermaNet bed net, would likely restrict the movements of larger nymphs and adults towards both a host and aggregation sites. Therefore, we suspect that these larger stages would likely become trapped and harbor within the bed net due to their inability to leave once fully fed. Conversely, smaller bed bugs could readily transit through the bed net and could aggregate in safer locations away from the host. Notably, the much larger mesh holes of the Olyset net would allow all bed bug life stages to readily pass through the bed net.

In common usage, however, bed nets are frequently not fully tucked under the mattress, and may dangle, even touching the bed frame and floor. In this case, the bed net may not serve as an effective barrier between the host and bed bugs. Instead, the LLIN may represent a walkway, connecting bed bugs from remote aggregations around the home to the host, independently of whether passage through the bed net is required or not. Under both scenarios, we envisage frequent interactions of bed bugs with LLINs, which are expected to select for pyrethroid resistance in the bed bug population. Further research is needed to understand how LLINs are used in the field, whether people change their use of LLINs when bed bugs proliferate and how LLINs with different mesh hole dimensions, active ingredients, or the incorporation of insecticide synergists modify the foraging behavior of bed bugs.

Pyrethroid resistance in bed bugs and mosquitoes

The parallel evolution of adaptive responses to insecticides in mosquito disease vectors and bed bugs is not new. DDT, an organochlorine that shares the voltage-gated sodium channel target site with pyrethroids, has been widely used in vector control on a global scale [41]. DDT selected for

various resistance mechanisms in both mosquitoes and bed bugs, and preadapted both for resistance to more recent use of pyrethroids, including in outdoor sprays (for mosquitoes, but not bed bugs), IRSs, and LLINs. Recent research has shown that exposure of *Aedes* and *Anopheles* populations to DDT has preselected for the evolution of multiple pyrethroid resistance mechanisms, including mutations that reduce target-site sensitivity to the insecticide, and metabolic detoxification [42-44]; similar mechanisms are likely to evolve in bed bug populations in the same communities. Together with the behavioral results presented here, these patterns strongly suggest that frequent exposure to pyrethroids in vector control programs, likely would select for pyrethroid resistance in bed bugs.

We posit that unintentional selection on bed bugs might be more intense than on target mosquitoes for four main reasons. First, all life stages of the hemimetabolous bed bug are exposed to pyrethroids, whereas only adults, and mainly adult female mosquitoes are targeted indoors. Second, mosquitoes are often repelled before settling on LLINs, due to the contact- and potential spatial repellency of LLINs, as well as the presence of other spatially repellent vector control tools, including mosquito coils [45]. Indeed, some LLINs (e.g., Olyset) are designed with large enough mesh holes that mosquitoes could pass through, but changes in mosquito behavior associated with repellency effectively prevent mosquitoes from doing so [46]. Therefore, direct and prolonged contact of mosquitoes with LLINs appears to be minimal. While mosquitoes may evolve olfactory adaptations that drive adaptive behavioral polymorphisms, leading to loss of LLIN repellency, our results suggest that bed bugs are unrepelled, or minimally repelled by pyrethroids in LLINs, regardless of resistance status [47]. While some insecticide-susceptible bed bugs were killed during their passage through LLINs, many survived, and none of the pyrethroid-resistant bed bugs died, suggesting that LLINs would impose strong selection for

resistance to pyrethroid insecticides. It is important to note that, to our knowledge, there are no pyrethroid-susceptible bed bug populations outside of laboratory colonies. Therefore, field populations of bed bugs are expected to more readily survive the brief interactions with LLINs that were imposed in our bioassays.

Third, related to the previous argument, adult mosquitoes fly and are relatively short-lived. In response to repellents, females may readily fly away, and may even egress the home in search of a host. In contrast, bed bugs are long-lived, wingless, and live strictly indoors. Therefore, bed bugs must find a blood meal within the home. Even if repelled by LLINs, bed bugs would remain in the home, capable of withstanding long periods of starvation, while searching for gaps in the LLIN-protection of the host. Finally, all these bed bug traits – dependence of all life stages on blood, wingless adults, low mobility, long-lived, resilience to starvation – contrast with mosquito traits and drive dramatically different population genetic structures in both insect taxa. Bed bug populations are highly inbred, often starting from small propagules of genetically related individuals [19]. Strong selection by LLINs and IRSs, especially on DDT-preadapted bed bugs, would quickly fix resistance alleles within a home population, eliminating reservoirs of susceptible individuals. This progression toward pyrethroid resistance would likely advance even in the face of fitness costs associated with specific resistance traits. In contrast, large outdoor reservoirs of insecticide susceptible mosquitoes, coupled with high mobility of adult females, would slow the evolution of pyrethroid resistance, especially if resistance mechanisms incur significant fitness costs. Overall, we suggest that LLINs and IRSs, which are designed to target mosquitoes, likely impose much stronger selection for the evolution of pyrethroid resistance in bed bugs than in their intended targets.

Constraints, limitations, and pertinence to C. hemipterus

This investigation represents a narrowly focused study in the laboratory, meant to generate hypotheses that can be further tested in the field within LLIN-based malaria interventions. Therefore, we used small sections of bed nets to assess the minimal interactions of bed bugs with bed nets. We would expect that large bed bugs that attempt to pass through a LLIN with small mesh holes will likely walk on the net for much longer time than in our behavioral assays, and thus they would be exposed to much higher doses of insecticides. As mentioned already, although our bioassays assessed bed bug passage through bed nets in two contexts – host-seeking to blood feed and aggregation-seeking – we made no assumptions about the frequency of these behaviors during the months-long lifetime of bed bugs.

We evaluated the responses of only two populations of *C. lectularius* representing two extreme phenotypes – a highly insecticide-susceptible reference strain that has been reared in a laboratory setting for five decades, free of exposure to insecticides, and a highly pyrethroid-resistant strain. It is unlikely that highly insecticide susceptible bed bug populations exist outside laboratory settings, due to global widespread pyrethroid use and documented resistance [31, 48]. While the highly resistant strain is typical of bed bugs collected globally in residential settings, the magnitude and distribution of pyrethroid resistance in remote villages in malaria endemic regions are yet to be thoroughly documented. Additionally, intraspecies level differences in host-seeking, feeding, aggregation behavior, and mechanisms of insecticide resistance could arise in bed bug populations based on local selection and adaptations. Evolutionary trade-offs between insecticide resistance and various life history traits, including behaviors, have been documented in many insect species, including bed bugs [49, 50]. It is possible that lower feeding and

aggregation responses, as we observed in the resistant bed bugs, represent such adaptive trade-offs that minimize movement and interaction with pesticides.

There is broad overlap in the distribution and incidence of *C. lectularius* and *C. hemipterus* in malaria endemic regions. While our findings broadly apply to *C. hemipterus*, this species possesses unique morphological and behavioral characteristics that might lessen the barriers offered by LLINs and exacerbate the adverse interactions of humans with bed bugs within LLINs. *C. hemipterus* bed bugs are more adept climbers than *C. lectularius* and are overall smaller at each life stage [51, 52]. Unlike *C. lectularius* in the northern hemisphere, present day exposure of *C. hemipterus* to both DDT and pyrethroids likely continues to select for high resistance to the most common active ingredients used in LLINs.

Conclusion: Potential burden of bed bugs on malaria control

Our research demonstrated that new commonly used LLINs failed to prevent unfed bed bugs from passing through bed nets to obtain a blood meal and fed bed bugs from passing through in response to aggregation stimuli. Repeated lethal and sub-lethal exposure of bed bugs to LLINs would rapidly eliminate susceptible bed bugs and favor individuals with emergent resistance mechanisms. These results suggest that despite proper use of LLINs, pyrethroid-resistant bed bugs would proliferate. Thus, our findings support the concerns of recent research in Sub-Saharan Africa, where the presence of pyrethroid-resistant bed bugs lead to LLIN abandonment, misuse, and failure to regularly reimpregnate the bed net with insecticide [24, 26, 27, 30]. Recent research has sought to address issues of stalled progress in the fight against malaria, with a focus on bio-efficacy and a perspective beyond insecticide resistance [53, 54]. We propose that insecticide-resistant secondary pest populations, namely bed bugs, should be investigated as an

additional potential factor contributing to stalled malaria control programs. Our findings also underscore the urgent need to invest in, reevaluate and innovate new designs of LLINs with a shared goal of effective mosquito and bed bug control.

Abbreviations

AI	Active ingredient
DDT	Dichlorodiphenyltrichloroethane
IRS	Indoor residual spray
LLIN	Long lasting insecticide-treated net
VBD	Vector borne diseases
WHO	World Health Organization

Declarations

Ethics approval and consent to participate

Bed bugs were fed on heparinized human blood supplied by the American Red Cross (IRB #00000288 and protocol #2018-026). The artificial feeding system was housed in a North Carolina State University-approved BSL-2 facility (Biological Use Authorization #2020-09-836).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors Contributions

Both authors worked collaboratively in the design of all bioassays used as part of this research. CCH performed all data collection, analysis, and interpretation. CCH wrote the primary draft of this manuscript, and then both authors contributed to the development of the final manuscript. Both authors have read and approved the final manuscript.

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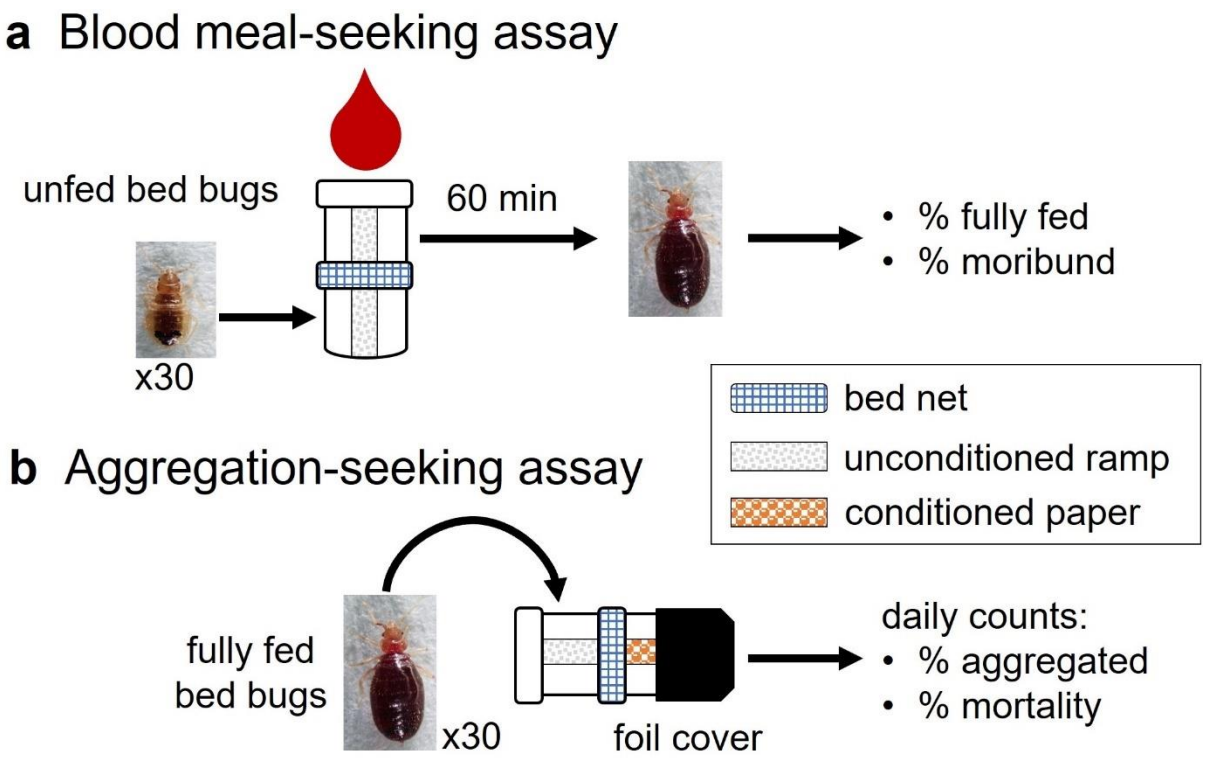

Fig. 2.1. Graphical representation of two behavioral assays designed to assess the interactions of two *C. lectularius* strains with commonly used bed nets. The bioassays quantified two distinct bed bug behaviors, namely blood meal (host)-seeking behavior of unfed bed bugs (**a**) and aggregation-seeking behavior of freshly fed bed bugs (**b**). Both assays assess passage of bed bugs through various bed nets in response to host cues and aggregation stimuli, respectively. Note that the aluminum foil in **b** completely covers the aggregation jar (see **Fig. 2c**) but is truncated in this illustration to show the conditioned (feces- and aggregation pheromone-impregnated) section of file folder paper within the jar.

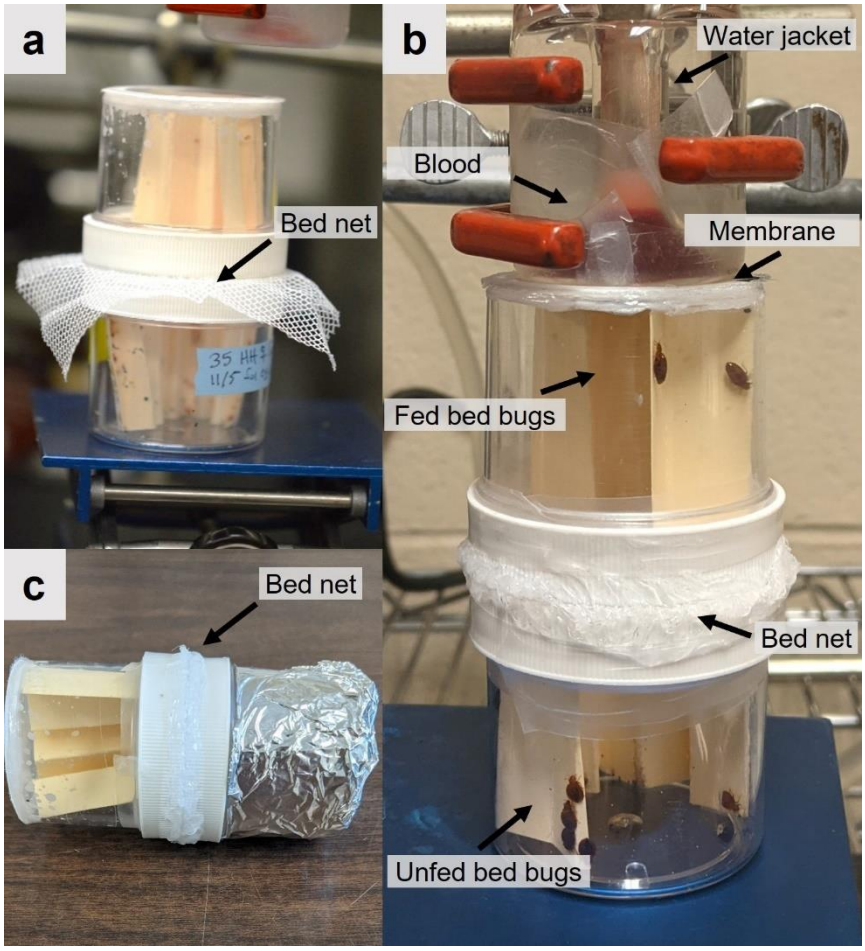

Fig. 2.2. Photos showing the two-jar bed net assembly used in the two behavioral assays. The two-jar assembly before the sandwiched bed net was trimmed (**a**). The blood meal-seeking assay in its vertical orientation, showing unfed *C. lectularius* at the starting point in the bottom jar and fed bed bugs in the top jar that abuts the blood feeder (**b**). The aggregation-seeking assay in the horizontal orientation, with fed bed bugs placed in the exposed jar (left) and the target aggregation jar (right) containing a section of conditioned (feces- and aggregation pheromone-laden) file folder paper and darkened with aluminum foil (**c**).

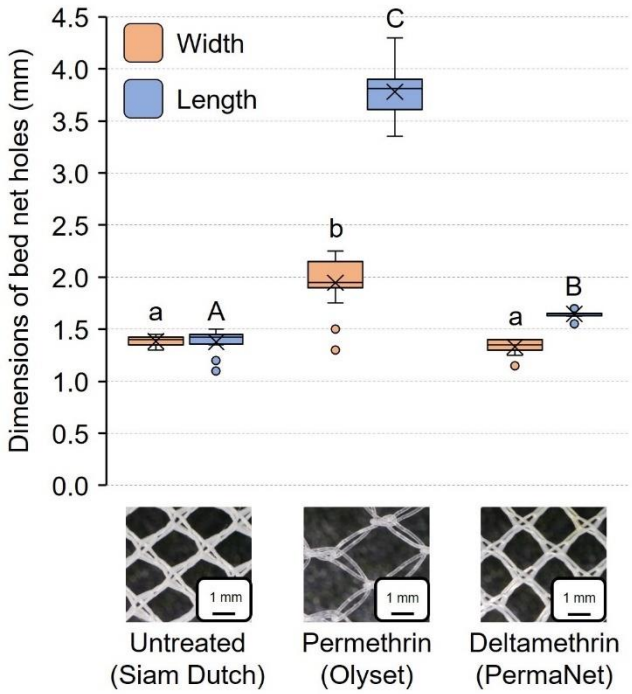

Fig. 2.3. Comparisons of the dimensions of mesh holes in bed nets used in this study. The width (orange) and length (blue) of 20 randomly selected holes were measured for each of the three bed nets. The mean is represented by x within each box plot. Photos of each net are shown with the same scale bars (1 mm). The widths of bed nets that share lower case letters are not significantly different from each other, and the lengths of bed nets that share upper case letters are not significantly different from each other (ANOVA, Tukey's HSD, $P > 0.05$).

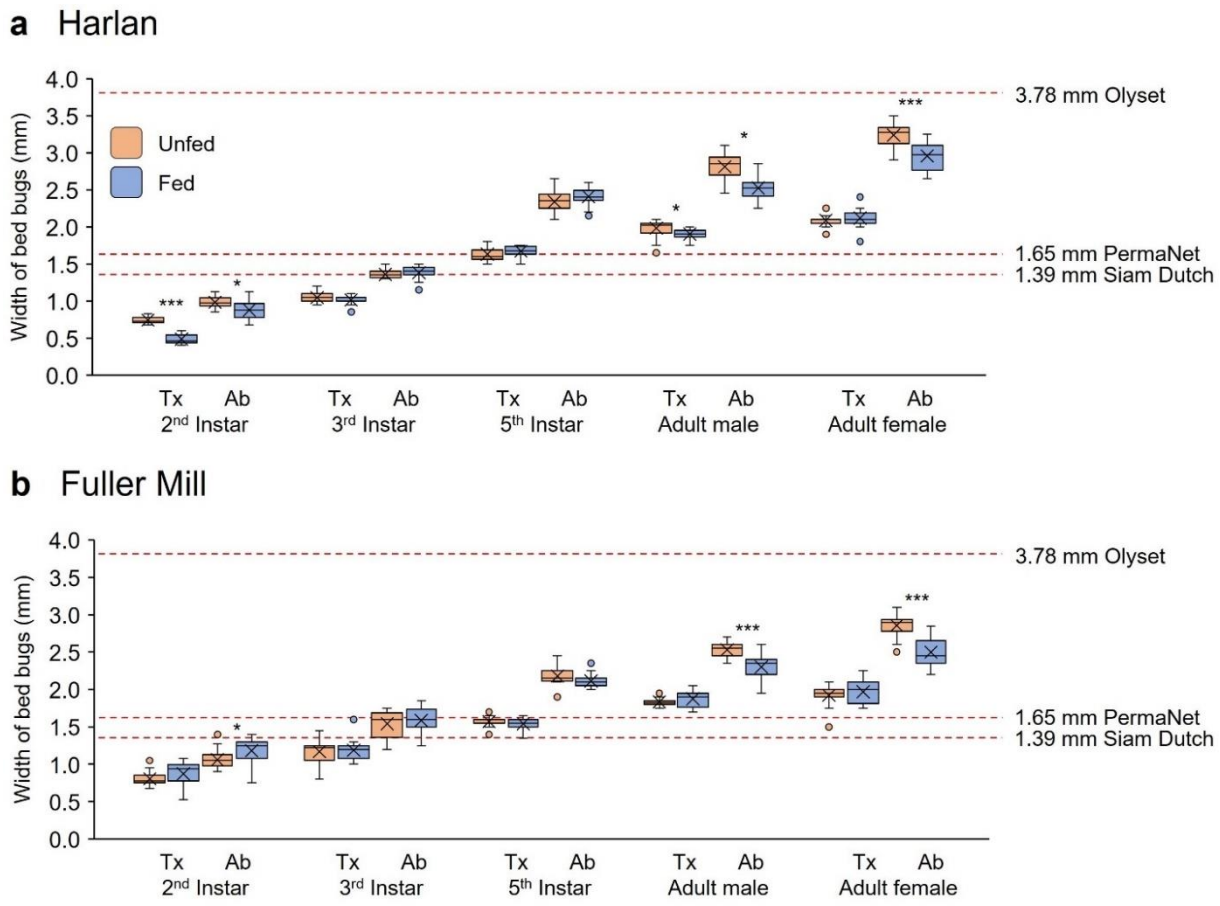

Fig. 2.4. Comparisons of the thoracic and abdominal widths of unfed and fed *C. lectularius* of two strains. The thoracic (Tx) and abdominal (Ab) widths of 2nd, 3rd, and 5th instars, as well as adult males and females (20 bed bugs per stage) were measured at both unfed (orange) and fed (blue) states for both the Harlan (insecticide-susceptible, **a**) and Fuller Mill (pyrethroid-resistant, **b**) strains. The results show increases in both thoracic and abdominal widths throughout development, and general decreases in abdominal width in fed bed bugs. The mean is represented by x within each box plot. The average largest linear dimension of mesh holes in the three bed nets are represented by red dashed lines. Significant differences (Student's *t*-test) in width between fed and unfed bed bugs are denoted by *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

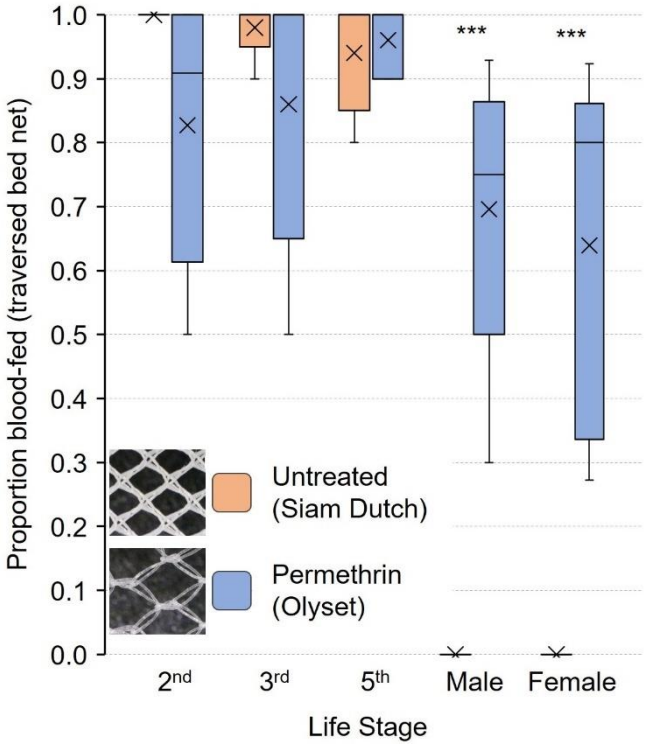

Fig. 2.5. Comparison of the blood meal-seeking assays of insecticide susceptible Harlan strain *C. lectularius* bed bugs of various life stages through the Siam Dutch untreated bed net and the Olyset permethrin-treated bed net. The mean is represented by x within each box plot. No significant differences were seen between the two bed nets in the proportion fed (traversed the bed net) for 2nd, 3rd, or 5th instars. However, significantly more males and females passed through the permethrin-treated bed net (larger mesh holes) than through the untreated bed net (Student's *t*-test, ***, $P < 0.0001$).

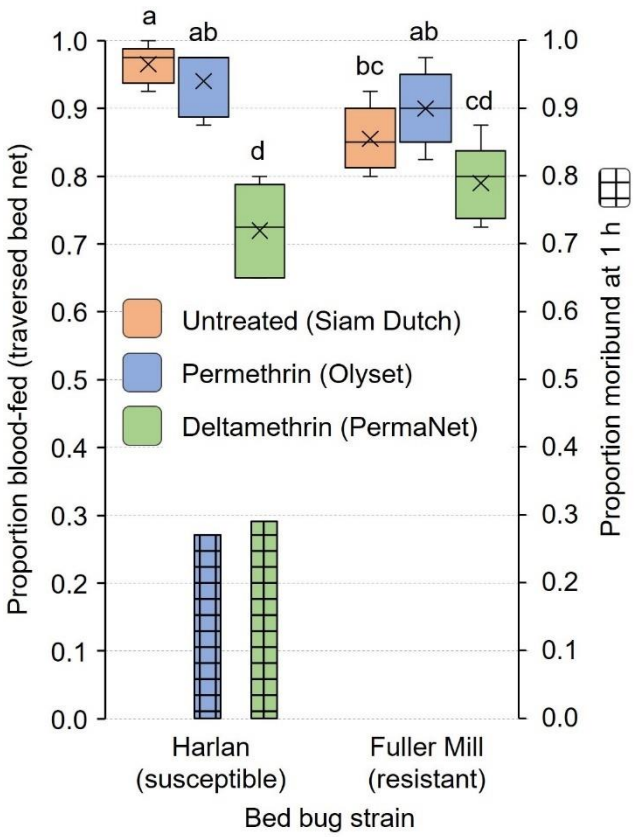

Fig. 2.6. Comparison of the blood meal-seeking assays and mortality of 2nd instar *C. lectularius* bed bugs belonging to the insecticide-susceptible Harlan strain, and the highly pyrethroid-resistant (resistance ratio >1,000) Fuller Mill strain. Comparisons were made between the two strains, as well as within strains to assess the impact of pyrethroid resistance on blood meal-seeking behavior. The mean is represented by x within each box plot. Treatments that share lower case letters are not significantly different from each other (ANOVA, Tukey's HSD, $P > 0.05$). Significantly lower proportions of blood-fed bed bugs were observed when bed bugs had to traverse deltamethrin-treated (PermaNet) bed nets. At 1 h, some Harlan (susceptible) bed bugs were moribund in these assays and all moribund bed bugs died at 48 h, whereas no morbidity or mortality were observed in Fuller Mill (resistant) bed bugs.

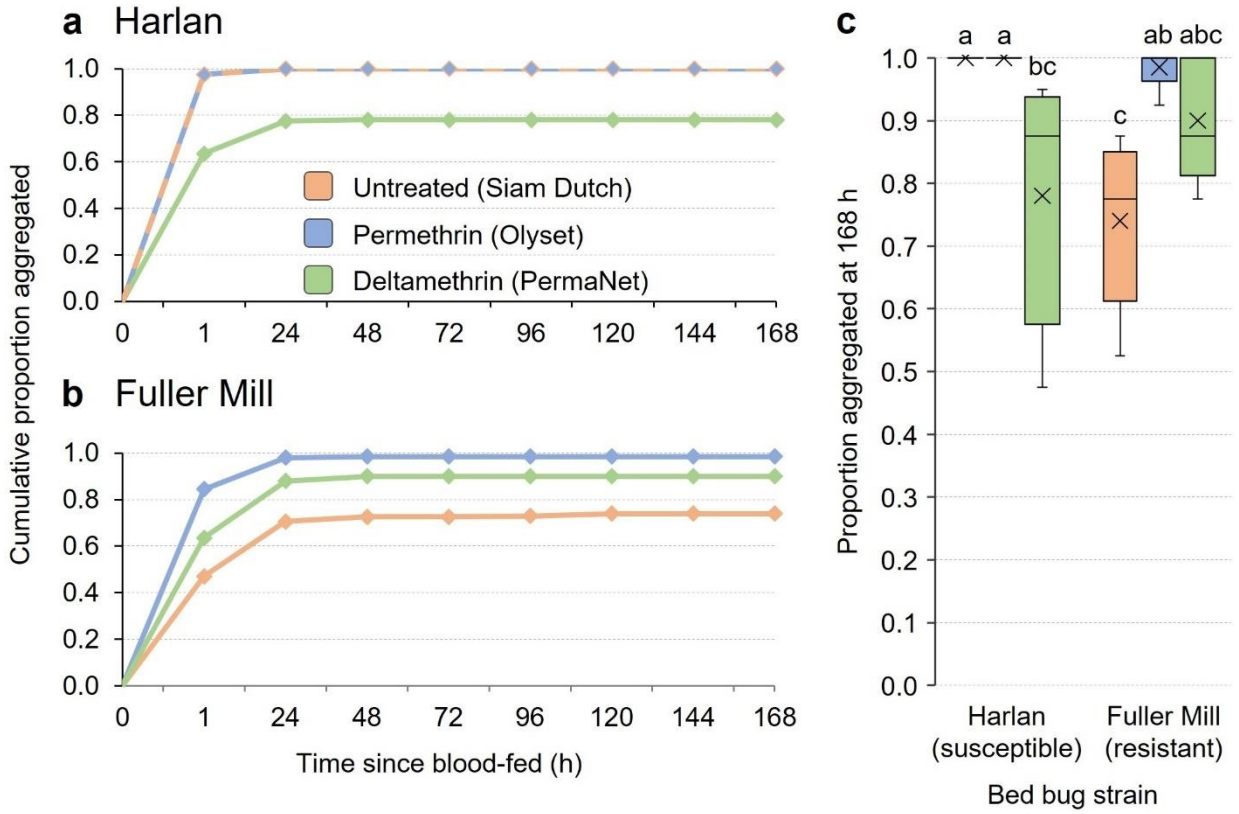

Fig. 2.7. Comparison of cumulative time-course of aggregation by 2nd instar *C. lectularius* bed bugs belonging to the insecticide-susceptible Harlan strain (**a**), and highly pyrethroid-resistant (resistance ratio >1,000) Fuller Mill strain (**b**). Assays were conducted with untreated (Siam Dutch, orange), permethrin-treated (Olyset, blue), and deltamethrin-treated (PermaNet, green) bed nets over the course of 7 d post feeding, or until all the bed bugs traversed the bed net into the aggregation jar. In **a**, the untreated and permethrin-treated nets followed the same aggregation pattern, and so are represented by a dashed line of their respective colors. The majority of all observed aggregation occurred by 48 h for both strains, and Harlan bed bug aggregation was only impeded over the course of this assay by the deltamethrin-treated LLIN. Fuller Mill bed bugs never achieved 100% aggregation through any net, with the untreated net having the largest impeding effect. Statistical comparisons of the cumulative aggregation at 196 h (**c**). The mean is represented by x within each box plot. Treatments that share lower case letters are not significantly different from each other (ANOVA, Tukey's HSD, $P > 0.05$). Significantly fewer Harlan bed bugs passed through the deltamethrin-treated LLIN and aggregated, and significantly fewer Fuller Mill bed bugs passed through the untreated net than the permethrin-treated LLIN.

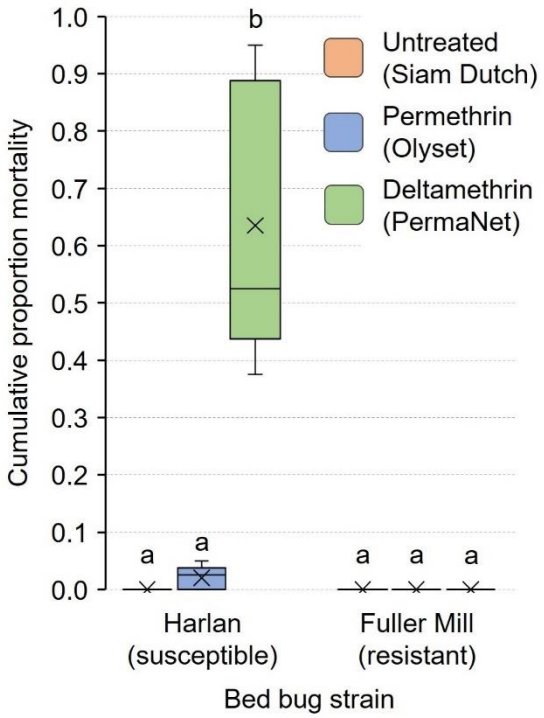

Fig. 2.8. Cumulative 7-days mortality of 2nd instar *C. lectularius* bed bugs in the aggregation-seeking assay. Fully fed insecticide-susceptible Harlan strain bed bugs and the highly pyrethroid-resistant (resistance ratio >1,000) Fuller Mill bed bugs were challenged to pass through three different bed nets to reach an aggregation site. The mean is represented by an x within each box plot. Significantly higher mortality in Harlan bed bugs was seen only when they interacted with the deltamethrin-treated LLIN (Wilcoxon signed-rank test, **, $P = 0.0079$).

Table 2.1. Characteristics of bed nets used in this study, based on manufacturer specifications

LLIN name	Manufacturer	AI (g/kg)[a]	Denier[b]	Mesh size (holes/cm^2)	Yarn type
Untreated	Siam Dutch	Not applicable	≥100	~24	Polyester Multifilament
Olyset	Sumitomo Chemical	Permethrin (20)	≥150	~5.28	Polyethylene Monofilament
PermaNet 2.0	Vestergaard	Deltamethrin (~1.6)	≥100	~24	Polyester Multifilament

a Concentration of active ingredient (AI).
b A measurement of the weight of yarn (g) per 9,000 meters of the yarn used in net production.

Table 2.2. Two-way ANOVA results for blood meal-seeking assays

Experiment	Term	*df* (Model, Error)	*F*-value	*P*-value
Multi-stage blood meal-seeking assay	Model	9, 40	33.17	<0.0001
	Stage	4	49.89	<0.0001
	LLIN	1	20.80	<0.0001
	LLIN*Stage	4	19.55	<0.0001
Single-stage blood meal-seeking assay	Model	5, 24	13.55	<0.0001
	Strain	1	4.95	0.0357
	LLIN	2	24.15	<0.0001
	LLIN*Strain	2	7.24	0.0035

Table 2.3. Two-way ANOVA results for aggregation-seeking assays at 1 h, 24 h, and 168 h.

Time points	Term	*df* (Model, Error)	*F*-value	*P*-value
1 h	Model	5, 24	2.71	0.0446
	Strain	1	3.23	0.0849
	LLIN	2	1.27	0.2993
	LLIN*Strain	2	3.89	0.0345
24 h	Model	5, 24	11.53	<0.0001
	Strain	1	7.89	0.0097
	LLIN	2	11.724	0.0003
	LLIN*Strain	2	13.17	0.0001
168 h	Model	5, 24	10.64	<0.0001
	Strain	1	4.56	0.0431
	LLIN	2	10.98	0.0004
	LLIN*Strain	2	13.33	0.0001

CHAPTER 3

Repellency of DEET During Host-Seeking Behavior of Bed Bugs (Hemiptera: Cimicidae) in Binary Choice Olfactometer Assays

(This work was published in *Journal of Medical Entomology*: **Hayes CC**, and **Schal C.** 2024. Repellency of *N*,*N*-diethyl-3-methylbenzamide (DEET) during host-seeking behavior of bed bugs (Hemiptera: Cimicidae) in binary choice olfactometer assays, *Journal of Medical Entomology*, 2024;, tjae073, https://doi.org/10.1093/jme/tjae073)

Abstract

The bed bug (*Cimex lectularius* L.) is one of the most prolific and burdensome indoor pests, and suppression of bed bug populations is a global priority. Understanding bed bug behavior is important to the development of new tactics for their control. Major gaps exist in our understanding of how host cues, insecticide resistance, and exposure modality impact the repellency of formulated products to bed bugs. Here, we validate the use of a binary choice olfactometer for assessing bed bug repellency behaviors using N,N-diethyl-3-methylbenzamide (DEET) in a dose-dependent manner, while considering the role of host-associated stimuli (with vs. without CO_2), exposure modality (olfactory vs. olfactory and contact), and resistance status (susceptible vs. resistant) on repellency. We observed that host-seeking insecticide susceptible bed bugs were repelled only when olfactorily exposed to high concentrations of DEET. However, exposure to DEET by contact repelled insecticide susceptible bed bugs at 100-fold lower dose of DEET. Further, we demonstrate for the first time that insecticide resistant bed bugs were significantly more responsive to DEET than susceptible bed bugs. We conclude that the 2-choice olfactometer is an effective tool for assessing behavioral responses of bed bugs to spatial and contact repellents.

Key words: bed bug, DEET, repellency, insecticide resistance, behavior

Introduction

The indoor environment is afflicted by several key arthropod pests, with species that are highly adapted to human-built structures being the most prevalent and persistent. Of these, bed bugs (*Cimex lectularius* L. and *Cimex hemipterus* F.) (Hemiptera: Cimicidae), which have resurged globally over the past two decades, are arguably the most difficult to control, due in large part to the widespread emergence of insecticide resistance (Dang et al. 2017, Lewis et al. 2023) . Bed bugs are obligatorily hematophagous but are not known to vector pathogens. Nonetheless, their bites are associated with allergic responses ranging from mild itching to painful infected lesions (Doggett et al. 2012, Hwang et al. 2018). Additionally, bed bugs disseminate in their feces high amounts of histamine, an immune modulator known to cause potentially severe respiratory response in vulnerable individuals (DeVries et al. 2018). The risk of these negative health outcomes increases alongside the burden of infestation, often arising from a combination of small founding propagules and failed eradication efforts (Saenz et al. 2012).

In response, a great deal of recent research has focused on the elucidation of resistance mechanisms in an effort to better inform product development, and the evaluation of new pest control tactics (Romero et al. 2007, Zhu et al. 2010, Lilly et al. 2016, Romero and Anderson 2016, Vander Pan et al. 2019, Dang et al. 2021, Ashbrook et al. 2022). Alongside these efforts, researchers have worked on assessing various natural and synthetic repellents against bed bugs in efforts to develop products that might disrupt host- and aggregation-seeking behaviors (Kumar et al. 1995, Wang et al. 2013, González-Morales et al. 2021a, Krüger et al. 2021, Shi et al. 2021). An example of such use is the impregnation of mattress covers with pyrethroid insecticides – similar in goal to the widespread use of long-lasting insecticide treated bed nets (LLINS) in vector control programs – to disrupt host seeking behaviors (Wilson et al. 2020).

In general, LLINs are weaved with large-holes throughout to facilitate air circulation, while relying on one or multiple active ingredients (AIs) impregnated into the net to repel and kill host-seeking mosquitoes and sandflies (Karunamoorthi 2011). In fact, it has been shown that LLINs provide ancillary benefits by controlling bed bugs and other domiciliary pests, and therefore communities rely heavily on these benefits in the absence of reliable alternatives (Temu et al. 1999, Malede et al. 2019). Despite this wide reliance, however, little is known about the interactions and overlap between bed bugs and LLINs. We have previously demonstrated that bed bugs readily penetrate commonly deployed LLINs, suggesting that LLINs may fail to repel host- and aggregation-seeking bed bugs, and may impose heavy selection pressure on bed bug populations for insecticide resistance (Hayes and Schal 2022). To date, no formal analysis of the repellency of LLINs to bed bugs exists. Although these products are not designed to target bed bugs, communities rely on them for bed bug control, warranting investigation of these interactions (Malede et al. 2019). In pursuit of this, we adapted and validated a forced-air binary choice olfactometer system to assess the impacts of known and putative repellents on bed bug host-seeking behaviors.

To date, bed bug repellency assays have included the application of repellent compounds or formulated products to exposed host skin in an effort to disrupt bed bug feeding behaviors, barrier applications of products, including insecticides, to disrupt host-seeking or aggregation-seeking, and comparisons of aggregation preferences on treated vs. untreated harborages (Kumar et al. 1995, Moore and Miller 2006, Wang et al. 2013, Liu et al. 2014, Anderson et al. 2018, Vander Pan et al. 2019, Krüger et al. 2021). Many of these studies have relied on DEET (N,N-diethyl-3-methylbenzamide) – the "gold standard" repellent known to be highly efficacious against both flying and crawling arthropods – as a positive control to validate the assay system

(Katz et al. 2008, Bissinger and Roe 2010, Chen-Hussey et al. 2014, Andreazza et al. 2021). It has also been shown that DEET remains highly repellent to insecticide resistant pests, with some preliminary evidence of this in bed bugs (Leal 2014, Liu et al. 2014, Mengoni and Alzogaray 2018). Given the reliance on and efficacy of DEET, it is surprising that large gaps exist in our understanding of how DEET influences bed bug host-seeking behaviors. For example, we are not aware of any dose-response studies of DEET repellency in bed bugs, or how various host-associated cues and various sensory modalities affect the repellency of DEET to bed bugs.

Therefore, in preparation for a formal assessment of whether bed bugs are repelled away from LLINs and AIs associated with LLINs, we developed and validated a binary choice olfactometer. In this paper we quantify the dose-dependent olfactory repellent effects of DEET on host-seeking bed bugs. We also compare responses to DEET in susceptible and multi-AI-resistant bed bugs, and the effects of host-associated cues (with vs. without CO_2) and sensory modalities (olfactory only vs. olfactory and contact repellency) on bed bug responses to DEET.

Materials and Methods

Colony Maintenance and Feeding

Two laboratory-maintained strains of *C. lectularius* were used in this study. The Harold Harlan strain (HH) (also known as Ft. Dix) is a commonly used insecticide-susceptible reference strain. It was collected at Fort Dix, New Jersey (USA) in 1973 and has not been challenged with insecticides since collection. The HH strain was maintained on a human host until December 2008, then, in our lab, on defibrinated rabbit blood until July 2021 and on human blood thereafter. The Fuller Mill Road strain (FM) was collected from a residence in High Point, North Carolina (USA) in 2017, and was maintained in our lab on defibrinated rabbit blood until July

2021 and on human blood thereafter. The FM strain is highly resistant to pyrethroids (González-Morales et al. 2022) and moderately resistant to fipronil (González-Morales et al. 2021b).

At time of use, both strains were maintained at 35–45% relative humidity, 25ºC on a 12:12 (L:D) h cycle and fed weekly on heparinized human blood (supplied by the American Red Cross under IRB #00000288 and protocol #2018-026). We used an artificial feeding system, which has been previously described (Sierras and Schal 2017, Hayes and Schal 2022). The feeding system was housed in a North Carolina State University-approved BSL-2 facility (Biological Use Authorization # 2020-09-836). Between feeding sessions, the glass feeders were sanitized with 7.5% sodium hypochlorite and 95% ethanol, and air-dried. Only adult females were used in all assays due to their need to obtain a bloodmeal between each oviposition cycle and thus high motivation to orient towards a potential host. Within 48 h post-feeding females were separated from colony jars into groups of 20–30 for a 10–14 d starvation period. Since the females were of unknown ages and likely mated within the colony, at several points during this period, but not within 24 h of the assay, groups of females were moved onto clean folder paper in clean vials (20 ml) to remove all eggs. Individual bed bugs were used for a single bioassay, and then discarded.

Human Odor Preparation

All human odors used as part of this research were collected from the primary researcher (CCH) following a previously validated and approved SOP for human skin swab collection (NCSU IRB Protocol 14173). In short, no alcohol, spicy, or pungent foods were consumed within at least 24 h of the odor collection. No sooner than 2 h before collection, CCH showered using Cetaphil ultra gentle body wash (Galderma, Fort Worth, TX) and no shampoo. No deodorant was applied, and

no strenuous activities performed. Hourly, between 2–10 h after showering, CCH cleansed his hands with water (no soap) and once dried, a filter paper (#1, 90 mm diameter, Whatman, Maidstone, United Kingdom) was used to swab a single forearm from wrist to elbow, armpit, and leg from ankle to knee for 15 s. A second filter paper was used to swab the corresponding regions on the other side of his body. Each filter paper was cut into 16 equal pie-shaped pieces (4 cm^2 each) stored in a glass vial at -20°C and used within one month.

DEET Dispenser Preparation

Individual squares (1.5 cm x 1.5 cm; 2.25 cm^2) or rectangles (1.5 cm x 3.0 cm; 4.5 cm^2) of #1 Whatman filter papers were placed on an aluminum foil and treated with either acetone (control) or DEET in acetone no more than 24 h prior to use and stored in glass vials (20 ml) at -20°C. Concentrations of DEET (PESTANAL analytical standard, Fisher Scientific, Waltham, MA) ranged from 0.001 µg/µl to 100 µg/µl. Squares were treated with a single 10 µl application of acetone or DEET and then air dried for 20 min, while each rectangle was folded and treated with two 5 µl applications (one per side; 2.25 cm^2) and dried for 10 min after each application. The total dose of DEET is reported throughout, but its concentration per square-cm may be derived by dividing by 2.25 and 4.5, respectively.

Assay Design

All assays were performed using glass Y-tube olfactometers as previously described (DeVries et al. 2019, Saveer et al. 2021), with minor modifications. Briefly, a vertically oriented 2 cm diameter glass Y-tube olfactometer with trunk length of 8.5 cm, arm-lengths of 6 cm, and glass odor pots of 5.5 cm inserted into the end of each arm, was connected to a forced air system (**Fig.**

3.1). Medical quality air (Airgas Healthcare, Radnor, PA) was passed through a humidifying jar at 200 ml/min and bifurcated into the olfactometer at the distal end of each odor pot so each arm received 100 ml/min, before rejoining at 200 ml/min in the common arm of the olfactometer. If assays used CO_2 (Airgas Healthcare, Radnor, PA), it was introduced alongside the already humidified air at 0.6 ml/min (regulated by a low-pressure regulator and a needle valve), which delivered approximately 3,000 ppm of CO_2. Plankton mesh (Wildco, Yulee, FL) was positioned in the center of the olfactometer and used as a walkway; it was replaced after no more than five replicate assays or for each assayed dose, whichever came first. Individual bed bugs were introduced to the assay via an uncapped releasing jar, and we recorded Activation (moving from the releasing jar into the common arm of the olfactometer), Choice (moving more than halfway up one of the assay arms (**Fig. 3.1A**) or crossing onto a DEET- or acetone-treated filter paper walkway (**Fig. 3.1B**)), and Preference (selected assay arm).

Prior to their introduction into the assay, each bed bug was placed in a releasing jar for 30 min of acclimatization, and then the releasing jar was attached to a conditioning port for 5–10 min to acclimate to assay airflow conditions. Insects were then introduced to the assay and given 5 min to walk up the common arm of the Y-tube and halfway up one of the assay arms (i.e., make a choice). All assays were run during the scotophase in a dark room at ~25°C, ~30–40% RH, with a dimmed red light to facilitate observations.

Two methods of odor introduction were used to assay olfactory-only and olfactory plus contact repellency. First, for olfactory repellency alone, both odor pots contained a 4 cm^2 pie-shaped piece of a skin swab (fresh swab for each assay) and one odor pot received a DEET-treated square filter paper while the other received a control (acetone-treated) filter paper (**Fig. 3.1A**). Second, for olfactory plus contact repellency, both odor pots contained a skin swab, and

each walkway in the side arms was affixed with either folded DEET-treated paper or a control (acetone-treated) paper (**Fig. 3.1B**). In both cases bed bugs were challenged to approach host cues (human odor with or without CO_2) in the presence or absence of DEET.

Statistical Analysis

All statistical analyses were conducted using SAS Enterprise Guide (v. 8.3, SAS Institute, Cary, NC), with $\alpha = 0.05$. Analyses of latency to activation and choice were done using Student's *t*-tests. For comparisons of percentage activation across treatment groups (total number activating by treatment as a percentage of total number assayed), we used a generalized linear model (GLM) followed by Tukey's HSD on arcsine square-root-transformed data. The percentage of HH bed bugs that made a choice (combined number making a choice at both arms of the olfactometer as the percentage of total number activated) across doses of DEET with and without CO_2 was analyzed using a Chi-square test followed by Holm's correction for multiple comparisons. The percentage bed bugs that made a choice across doses of DEET in assays involving both strains was arcsine square-root-transformed and analyzed using one-way ANOVA followed by Tukey's HSD within each strain, or using a GLM followed by Tukey's HSD between strains. Dose-dependent percentage preference (one arm as percentage of total that made a choice) was compared via individual Chi-square tests based on provided stimuli (CO_2 vs. no CO_2) in single-strain assays, and by strain in assays involving both HH and FM strains.

Results

Validation of the Olfactometer Assay with Host Cues

Using the olfaction-only assay (**Fig. 3.1A**) we evaluated the effectiveness of the olfactometer with human odor alone and with human odor plus CO_2 in the absence of DEET. In the positive control, where a human skin swab alone was presented in one arm of the olfactometer and a control filter paper in the other, 97.2% of bed bugs were activated, with 82.8% making a choice, of which 100% chose the human odor arm over the control arm ($\chi^2 = 24$, $df = 1$, $P < 0.01$) (**Fig. 3.2A**). The lack of side-bias in the olfactometer was confirmed with human odor (no CO_2) emanating from both arms of the olfactometer, resulting in 100% activation, with 76.2% of the bed bugs making a choice, and 50% choosing each arm of the olfactometer ($\chi^2 = 0$, $df = 1$, $P > 0.05$) (**Fig. 3.2A**). Thus, this olfactometer is appropriate for resolving olfactory preferences of bed bugs.

Similar results were obtained with the addition of CO_2 to human odor (**Fig. 3.2B**). In both treatment groups 100% of the bed bugs were activated and 78.3% and 81.3% made a choice in the positive control assays (odor plus CO_2 vs. CO_2-only) and side-bias assays (both arms with human odor plus CO_2), respectively. In the positive control treatment group, 100% of the bed bugs preferred the human odor plus CO_2 arm over the CO_2-only arm ($\chi^2 = 18$, $df = 1$, $P < 0.01$) and there was no evidence of side-bias when both arms emitted human odor and CO_2 ($\chi^2 = 0.692$, $df = 1$, $P > 0.05$). The high resolution and lack of side-bias of the olfactometer was further confirmed in all subsequent experiments that compared the olfactory-only preferences (**Fig. 3.3A**) and olfactory plus contact preferences (**Fig. 3.3B**) of two strains of bed bugs.

Responses to DEET in the Presence of Host Olfactory Cues

Using the olfaction-only assay (**Fig. 3.1A**) we assessed the effects of increasing doses of DEET on bed bug responses in the presence of human odor alone or human odor plus CO_2. Despite the presence of DEET, an overall high percentage of bed bugs (90.0–100%) activated in response to human odor alone across all but the highest assayed DEET dose (**Fig. 3.2A**). At 1,000 μg of DEET, significantly fewer bed bugs were activated (40.0%) in response to human odor alone (one-way ANOVA, $F = 9.60$, $df = 7,40$, $P < 0.001$; Tukey's HSD, $df = 40$, $P < 0.05$). However, the addition of CO_2 to human odor raised bed bug activation to 100% across all doses of DEET (**Fig. 3.2B**). Thus, higher quality host cues (host odor plus CO_2) stimulate bed bugs to overcome the repellency of DEET in search of a blood-host. Conversely, high concentrations of DEET are more effective at repelling bed bugs from lower quality host cues (host odor alone).

With human odor alone (**Fig. 3.2A**), the percentage making a choice declined significantly from 80% at 1 μg DEET to 10% at 1,000 μg DEET (Chi-square followed by Holms correction; $\chi^2 = 44.54$, $df = 6$, $P < 0.0001$). A significant decline in the percentage making a choice was seen at 10 μg ($P = 0.0304$), 100 μg ($P = 0.0102$), and 1,000 μg ($P < 0.0001$) DEET relative to the side-bias control. The addition of CO_2 to human odor (**Fig. 3.2B**) had varying effects, but overall increased the percentage of bed bugs that made a choice ($\chi^2 = 29.22$, $df = 6$, $P < 0.0001$), with a significant decline in the percentage making a choice only at 1,000 μg DEET ($P = 0.0105$). In both sets of assays, no significant effects on preference were evident at ≤10 μg DEET (**Fig. 3.2**). In the presence of 100 μg DEET, 100% of the bed bugs preferred the control arm of the olfactometer, regardless of host cue quality (human odor with CO_2 ($\chi^2 = 6$, $df = 1$, $P < 0.05$) or without CO_2 ($\chi^2 = 8$, $df = 1$, $P < 0.01$)). Likewise, in the presence of 1,000 μg DEET, 100% of the bed bugs preferred the control arm of the olfactometer (no DEET) regardless of

provided host cues (human odor with or without CO_2). However, only 2 of 20 bed bugs responded in the presence of host odor alone (**Fig. 3.2A**), whereas 4 of 20 bed bugs responded when CO_2 was added to host odor ($\chi^2 = 4$, $df = 1$, $P < 0.05$) (**Fig. 3.2B**). These findings demonstrate that bed bugs can overcome the presence of a spatial (olfactory) repellent in pursuit of host-emitted cues, except at remarkably high concentrations of the repellent.

Strain Variation in DEET Olfactory Repellency

To determine if variation in spatial repellency exists between strains, we compared the activation, latency, choice, and preference of the insecticide susceptible HH strain and the insecticide resistant FM strain at two doses of DEET, using olfactory-only assays (**Fig. 3.1A**). Based on the HH dose-response results with host odor and CO_2 (**Fig. 3.2B**), we used 10 μg DEET, at which HH bed bugs were not repelled, and 1,000 μg DEET, which significantly repelled all bed bugs that made a choice. In both strains, 100% of the bed bugs activated in all treatment groups. Further, a high percentage of bed bugs making a choice was seen in the positive controls of both strains (HH = 80.0%, FM = 81.8%) (**Fig. 3.3A**). However, whereas in HH bed bugs the percentage that made a choice significantly declined at 1,000 μg DEET to only 20.0% (one-way ANOVA, $F = 7.24$, $df = 3,15$, $P = 0.0031$; Tukey's HSD, $df = 15$, $P < 0.05$), 70.0% of the FM strain bed bugs made a choice with no significant differences among DEET doses (one-way ANOVA, $F = 0.68$, $df = 2,9$, $P = 0.5297$). Comparison of the two strains based on the percentages of bed bugs making a choice revealed an overall significant model (GLM, $F = 3.87$, $df = 6,24$, $P = 0.0077$), with only dose significantly affecting the percentage making a choice (Tukey's HSD, $F = 5.69$, $df = 3$, $P = 0.0043$). Neither strain alone ($F = 3.24$, $df = 1$, $P = 0.0843$) nor the interaction of dose and strain ($F = 2.46$, $df = 2$, $P = 0.1069$) significantly affected

the percentage making a choice (**Fig. 3.3A**). Specifically, no significant difference in the percentage making a choice was seen between the strains at 10 μg DEET (Tukey's HSD, t = -0.54, df = 24, P = 0.5965), but significantly more of the FM than HH bed bugs made a choice at 1,000 μg DEET (Tukey's HSD, t = -3.95, df = 24, P = 0.0006) (**Fig. 3.3A**). The results suggest that high aerial concentrations of DEET are more repellent to the insecticide resistant FM strain than to HH bed bugs in the presence of host cues.

In both the HH and FM controls, 100% of the bed bugs that made a choice were attracted to human odor and CO_2 (Chi-square test, HH: χ^2 = 18, df = 1, P < 0.01; FM: χ^2 = 9, df = 1, P < 0.01). The HH bed bugs showed no significant preference at 10 μg DEET, with 50% choosing each arm of the olfactometer (χ^2 = 0, df = 1, P > 0.05). However, they were significantly repelled by 1,000 μg DEET, with 100% choosing the host cues-only arm (χ^2 = 4, df = 1, P < 0.05) (**Fig. 3.3A**). In contrast, the FM bed bugs were significantly repelled at both doses (10 μg: χ^2 = 8.067, df = 1, P < 0.01; 1,000 μg: χ^2 = 14, df = 1, P < 0.01) (**Fig. 3.3A**). These results, taken alongside our observations that more FM bed bugs make a choice at high DEET concentrations, suggest that the FM strain might be more olfactorily sensitive to DEET than HH bed bugs, resulting in an increased excito-repellent response.

Strain Variation in Combined Olfaction and Contact Repellency of DEET

To further understand the repellent effects of DEET, we challenged host-seeking bed bugs to orient upwind toward host-associated cues in the presence of DEET volatiles (olfactory repellency) and then traverse a DEET-treated filter paper (contact repellency) using a modified assay design (**Fig. 3.1B**). The percentage of bed bugs that activated remained high (≥80%), but the addition of contact exposure reduced activation of HH bed bugs at the highest dose of DEET

(GLM, $F = 2.72$, $df = 9,36$, $P = 0.0157$) (**Fig. 3.3B**). Namely, the dose of DEET significantly affected activation ($F = 3.46$, $df = 5$, $P = 0.0118$), but neither strain ($F = 0.65$, $df = 1$, $P = 0.4259$) nor the interaction of strain and dose ($F = 2.42$, $df = 3$, $P = 0.0823$) had a significant effect. There was significantly lower activation of HH (80.0%) than FM (95.0%) bed bugs at 1,000 µg DEET (Tukey's HSD, $t = -3.63$, $df = 35$, $P = 0.0009$).

Further, high percentages of both HH and FM bed bugs preferred human odor in the positive control assays (no DEET) (**Fig. 3.3B**). The pattern within each strain was similar to that with olfactory stimulation with DEET. A significantly lower percentage of HH bed bugs reached the choice point when exposed to 1,000 µg DEET by olfaction and contact (40.0%) (one-way ANOVA, $F = 5.00$, $df = 4,21$, $P = 0.0054$; Tukey's HSD, $P < 0.05$), but FM bed bugs responded equally (87.5–100%) to these stimuli (one-way ANOVA, $F = 0.57$, $df = 3,14$, $P = 0.6433$) (**Fig. 3.3B**). Comparison between strains of dose-dependent percentage bed bugs that made a choice revealed an overall significant model (GLM, $F = 3.86$, $df = 8,35$, $P = 0.0024$). All three factors in the model significantly affected percentage that made a choice: dose ($F = 2.84$, $df = 4$, $P = 0.0386$), strain ($F = 13.33$, $df = 1$, $P = 0.0008$), and the interaction of dose and strain ($F = 4.22$, $df = 3$, $P = 0.0120$). As in the olfaction only assays, at 1,000 µg DEET, significantly more FM bed bugs made a choice (95.0%) than HH bed bugs (40%) (Tukey's HSD, $t = 3.33$, $df = 35$, $P = 0.0020$).

All bed bugs of both strains preferred to orient to the blend of human odor and CO_2 over CO_2 alone in the absence of DEET ($\chi^2 = 10$, $df = 1$, $P < 0.01$). Also, both strains of bed bugs showed no side preference in the presence of 1 µg DEET (HH: $\chi^2 = 0.222$, $df = 1$, $P > 0.05$; FM: $\chi^2 = 0.053$, $df = 1$, $P > 0.05$), suggesting that they did not perceive DEET by olfaction and contact at this concentration. Unlike in the olfaction-only assays, however, HH bed bugs were

significantly repelled by 10 μg DEET (80.0%) ($\chi^2 = 5.4$, $df = 1$, $P < 0.05$), and 100% of FM bed bugs oriented away from the DEET arm of the olfactometer. With both strains, 1,000 μg DEET repelled 100% of the bed bugs (HH: $\chi^2 = 8$, $df = 1$, $P < 0.01$; FM: $\chi^2 = 19$, $df = 1$, $P < 0.01$) (**Fig. 3.3B**), as in the olfaction-only assays (**Fig. 3.3A**). Overall, the combination of the olfactory (spatial) and contact repellency of DEET resulted in greater repellency of host-seeking bed bugs than olfactory repellency alone.

Analysis of Latency to Activation and Choice

To further assess the effect of host cue quality, doses of DEET, and strain on bed bug behavior, we compared the latency to activation and latency to making a choice across all treatment groups. The latency results did not follow a clear pattern, so they are presented as Supplementary Information. Considering the quality of host cues (odor only: **Fig. 3.2A**, or odor plus CO_2: **Fig. 3.2B**), the latency to activation was not significantly different across the controls and all doses of DEET (**Table 3.1**). Latency to making a choice was significantly lower with the addition of CO_2 in the side-bias assays (Student's *t*-test, $df = 31$, $P = 0.0033$) and at 1 μg of DEET (*t*-test, $df = 30$, $P = 0.0044$), and nearly so at 0.01 μg of DEET ($P = 0.0818$) (**Table 3.2**). In olfaction only assays there were no significant differences across the controls, both doses of DEET, or between assayed bed bug strains in latency to activation or choice. In olfaction plus contact assays, as in other experiments, activation latency did not appear to reveal consistent patterns (**Table 3.1**). As well, comparison of choice latencies did not reveal any useful insights, with significantly slower time to making a choice by FM in the positive control (*t*-test, $df = 37$, $P = 0.0321$), and significantly faster time to making a choice by FM at 1 μg DEET (**Table 3.2**).

Discussion

To our knowledge, this is the first study to assess the repellency of DEET to bed bugs (1) in dose-response assays of olfactory repellency, (2) in relation to varying the quality of host-associated cues, (3) by combining the effects of olfactory and contact repellency, and only the second study to consider the potential association between insecticide resistance and repellency of DEET to bed bugs (Vassena et al. 2019). We were able to accomplish this with a binary choice olfactometer that examines upwind orientation to attractive host cues and is highly sensitive to slight differences in the quality of olfactory stimuli that emanate from each of the two arms of the olfactometer. Briefly, we have shown that as the quality of host cues improves (human odor plus CO_2), so does the propensity of bed bugs to overcome the repellent while orienting toward the host odor. Further, we have shown that the combination of olfactory (spatial) and contact DEET repellency was more effective than olfactory repellency alone. Finally, we observed strain differences in bed bug responses to DEET which may be associated with insecticide resistance – resistant bed bugs were repelled at lower concentrations of DEET than susceptible bed bugs. Our finding that bed bugs are significantly less repelled by DEET in the presence of high-quality human-associated cues highlights the need to conduct repellency assays under more realistic conditions wherein repellents are challenged to disrupt the innate attraction of bed bugs to host or aggregation stimuli.

The use of binary choice olfactometers to elucidate mechanisms of spatial and contact repellency is common in research across insect orders, where either individuals or groups of insects are simultaneously exposed to stimuli and their orientation observed (Grieco et al. 2005, Zhu et al. 2015, Brito et al. 2021). Olfactometer-based assays have been pivotal in elucidating chemically mediated insect behaviors and influencing pest management decisions (Grieco et al.

2007, Roberts et al. 2023), but they have been underutilized in bed bug research. Herein, we have demonstrated the utility of binary choice olfactometers to assess the repellency of insecticides and behavior-modifying products to bed bugs, and their sensitivity to resolve changes in repellency in response to various cues, strain variations, and exposure modality.

Host-Associated Cues and DEET Repellency

Behavioral assays of repellency rely on effective attractants that stimulate directed orientation behaviors, or arrestants that cause the insect to cease activity; the repellent's effect at disrupting orientation or arrestment can then be quantified (Syed and Leal 2008, Roberts et al. 2023). In bed bug repellency assays, attractants have largely consisted of host cues (e.g., human odor, heat, CO_2) or aggregation cues (e.g., bed bug-conditioned harborages), with light often used as an aversive stimulus to drive bed bugs into the dark shelters (González-Morales et al. 2021a, Krüger et al. 2021, Hayes and Schal 2022). Few studies have sought to optimize the quality of attractants or assess the combinatory or synergistic effects of multiple attractants – especially of different sensory modalities – on bed bug orientation, while challenging bed bugs to overcome repellents embedded in the air stream that carries the attractants.

We found that the addition of CO_2 to human skin odor (that is, high quality host cues) significantly increased the number of bed bugs that oriented upwind despite high concentrations of DEET in the attractive air stream (**Fig. 3.2**). Although bed bugs ultimately chose the DEET-free arm of the olfactometer, their greater propensity to move toward an attractive host-associated chemical blend, despite high concentrations of DEET, may result in more frequent interactions with repellents in the field, increasing the risk that bed bugs might find gaps in repellent coverage and contact the host. Our results likely underestimate the ability of bed bugs

to overcome repellents in the presence of a host, because we did not consider body heat which is known to stimulate orientation, and did not optimize the skin swab or consider human variations in olfactory cues (DeVries et al. 2016). Overall, these findings suggest that the presence of multiple optimized host-associated cues may compromise the efficacy of repellents on host-seeking bed bugs. These observations highlight the importance of using effective attractants representative of field conditions, rather than blank (or solvent) controls in binary choice assays.

Insecticide Resistance and DEET Repellency

A large body of literature now exists on the relationship between pyrethroid resistance and accompanying changes in sensitivity to repellents. It has been demonstrated that while *kdr*-type mutations that affect activation of the sodium channel provide resistance to pyrethroids, at least two of these mutations had no effect on DEET repellency in *Aedes aegypti* L. (Diptera: Culicidae) (Andreazza et al. 2021). However, there is no consensus across insect orders; in some species insecticide resistance is associated with lower sensitivity to repellents, whereas in other species no clear associations were found (Wagman et al. 2015, Wu and Appel 2018, Fardisi et al. 2019). For example, a field-collected pyrethroid-resistant strain of *C. lectularius* from Argentina was found to have lower sensitivity to DEET than the insecticide susceptible Harlan (HH) strain (Vassena et al. 2019). In contrast, our assays indicate that the multi-AI-resistant FM strain was repelled at 100-fold lower dose of DEET than HH bed bugs (**Fig. 3.3A**), suggesting greater sensitivity to DEET.

The higher repellency at lower DEET concentrations was further confirmed in the combined olfaction and contact assays, where 100% of FM bed bugs made a choice (i.e., oriented upwind and entered either arm of the olfactometer) at 10 µg DEET, yet 100% avoided

the DEET-treated walkway at the same dose (**Fig. 3.3B**). We posit that the observed excito-repellent behavior, characterized by rapid movement, higher percentage making a choice, and consistent avoidance of DEET, supports the notion of higher DEET sensitivity in the FM strain. Further studies are needed with a range of resistant bed bug populations in which resistance mechanisms and target site mutations are characterized and associated with behavioral changes in sensitivity to repellents. Recent global surveys indicate that few, if any, insecticide susceptible *C. lectularius* populations exist in the field (Dang et al. 2017, Lewis et al. 2023). Therefore, it is important to understand the far-reaching impacts of widespread insecticide resistance on bed bug behavioral responses to repellents and repellent insecticides.

Olfactory and Contact Repellency of DEET

Assays of bed bug repellency to DEET have concentrated on contact repellency, rarely considering spatial (olfactory) repellent effects independently. For example, in Petri dish assays, a portion of the substrate is treated with a repellent, and the position of bed bugs is recorded over time (Wang et al. 2013, González-Morales et al. 2021a, Krüger et al. 2021). In this case, both contact and olfactory repellency contribute to assay outcomes, but their respective contributions cannot be disentangled. In contrast, olfactometers that use directional air flow assay olfactory repellency separately from contact exposure, consistent with the recent identification of DEET-sensitive olfactory receptor neurons in *C. lectularius* through single sensillum recordings (Liu et al. 2017). Representing the first known assays of this kind in bed bugs, our results demonstrate that the combined olfactory and contact exposure of bed bugs to DEET significantly elevated the repellency of DEET and required 10–100-fold less DEET for the expression of repellency in the HH strain relative to olfactory (spatial) repellency alone (**Fig. 3.3B**). We suggest that

investigating both exposure modalities, olfaction and contact, leads to a deeper understanding of bed bug behavioral interactions with repellents and insecticidal products, as volatilized chemicals are frequently encountered prior to contact with treated surfaces, and novel formulations may amplify or suppress olfactory repellency to enable more effective bed bug management.

Field Relevance and Conclusion

In summary, we have for the first time assessed the repellency of DEET to the common bed bug *C. lectularius* in a dose-dependent manner, considering the independent modality of olfaction-based exposure, the addition of contact exposure, the impact of differing host-associated stimuli, and the role of insecticide resistance. Despite the extensive use of synthetic repellents, essential oils, and repellent insecticides in bed bug control, large gaps remain in our understanding of bed bug sensitivity to repellents, and changes to repellency associated with the volatility of repellents, their mode of application, and insecticide resistance in bed bugs. Certainly, the purpose of these assays was not to suggest the use of DEET as a repellent product in bed bug control, although our work further elucidates the parameters of its use as a standard repellent in bed bug research. Instead, we used DEET as a reference repellent to demonstrate the utility of binary choice olfactometers to assess the repellency of field-applied products to bed bugs.

One such case, as already mentioned, is the widespread use of LLINs to protect sleeping humans from disease vectors, which has relied on the excito-repellent properties of pyrethroid insecticides. The deployment of LLINs in the same environment where bed bugs live (indoors, beds) to protect the same host that be bugs seek (humans) has imposed strong selection pressure on bed bugs, which have become significant pyrethroid-resistant domiciliary pests in malaria-endemic areas (World Health Organization 1968, Busvine and Pal 1969, Kweka et al. 2009,

Deku et al. 2021). Treated LLINs have been shown to disrupt mosquito host-seeking behaviors, but their effect on bed bug host-seeking behaviors is still unknown (Parker et al. 2015). Our assays with DEET will guide future work with LLINs to elucidate potential impacts of vector control on bed bug populations.

Declarations

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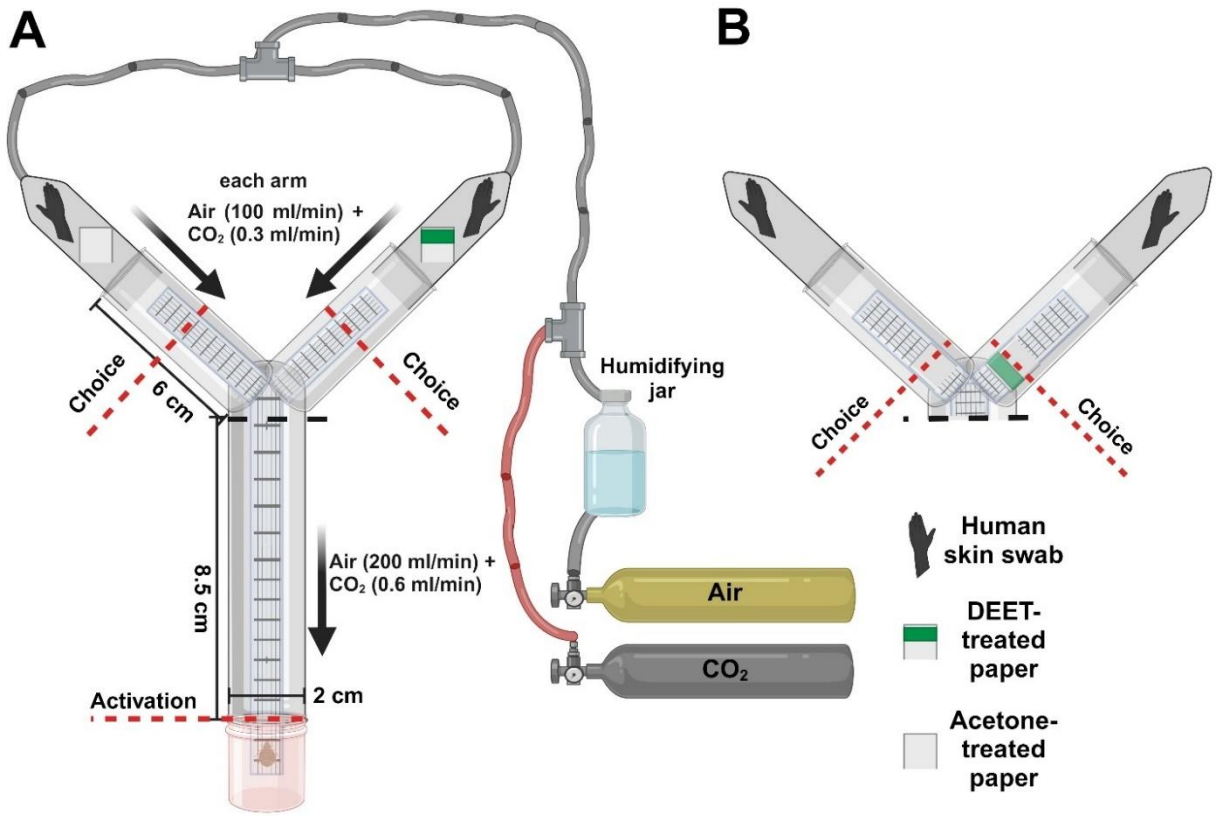

Fig. 3.1. Graphical representation of the binary choice olfactometer used to assess the behaviors of *C. lectularius* to varying doses of DEET. Assays were designed to test olfactory (spatial) repellency independently of contact exposure (**A**) and olfactory plus contact repellency (**B**). In both assay designs, host-seeking adult female bed bugs are attracted toward host-associated olfactory cues (human skin volatiles and CO_2) in humidified air. Behavioral responses to various concentrations of DEET were quantified by measuring percentage activation (bed bugs that entered the olfactometer / total bed bugs assayed); percentage choice (bed bugs that made a choice of either arm of the olfactometer / total bed bugs that activated); arm preference (percentage that chose the treatment vs. control arms); and latency (s) to activation and making a choice, respectively. DEET and human skin swab stimuli were prepared independently and introduced via separate filter papers. Human skin swabs were replaced after each replicate, and walkways were changed after either 5 replicates or between treatment groups, whichever came first.

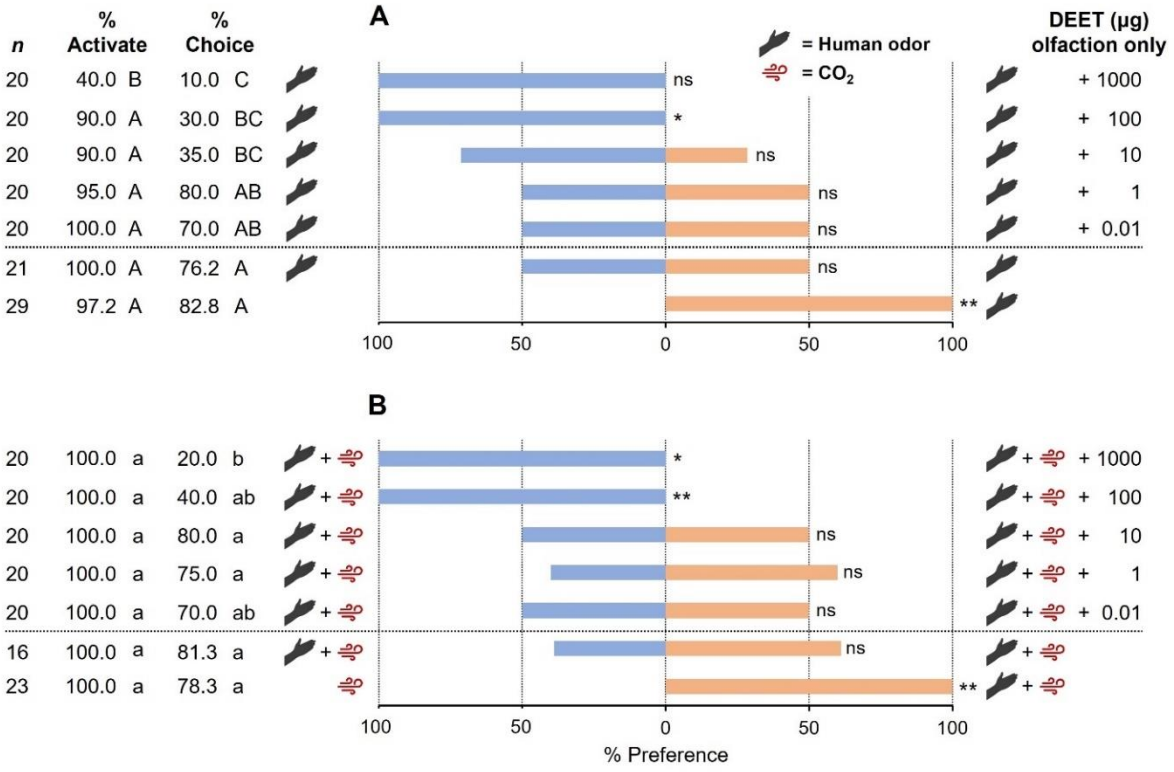

Fig. 3.2. Comparisons of olfactory-mediated behavioral responses of insecticide susceptible (HH strain) *C. lectularius* to various doses of DEET in the absence (**A**) or presence (**B**) of CO_2. Individual 10–14 d starved females were provided respective host-associated stimuli. The positive control (bottom treatment group in each graph) consisted of host cues (odor and/or CO_2) at only one arm of the olfactometer, and the olfactometer side-bias control (second treatment group from the bottom in each graph) had identical host cues at both arms. Activation was defined as the female leaving the release vial and entering the olfactometer, and aggregate activation (% Activate) is shown for each treatment group. The percentage of activated bed bugs that chose either arm of the olfactometer is shown as % Choice. The aggregate percentage choosing the subject stimuli, including DEET (right side, orange), and towards the control stimuli (left side, blue) are shown. Percentage Activate was compared within each graph and those that share case-specific letters are not significantly different (One-way ANOVA, Tukey's HSD). Likewise, % Choice was compared within each graph and those that share case-specific letters are not significantly different (Chi-square test, Holm's correction). Percentage Preference was compared independently within each treatment group (e.g., each dose) by Chi-square test, with asterisks representing significant differences denoted by *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

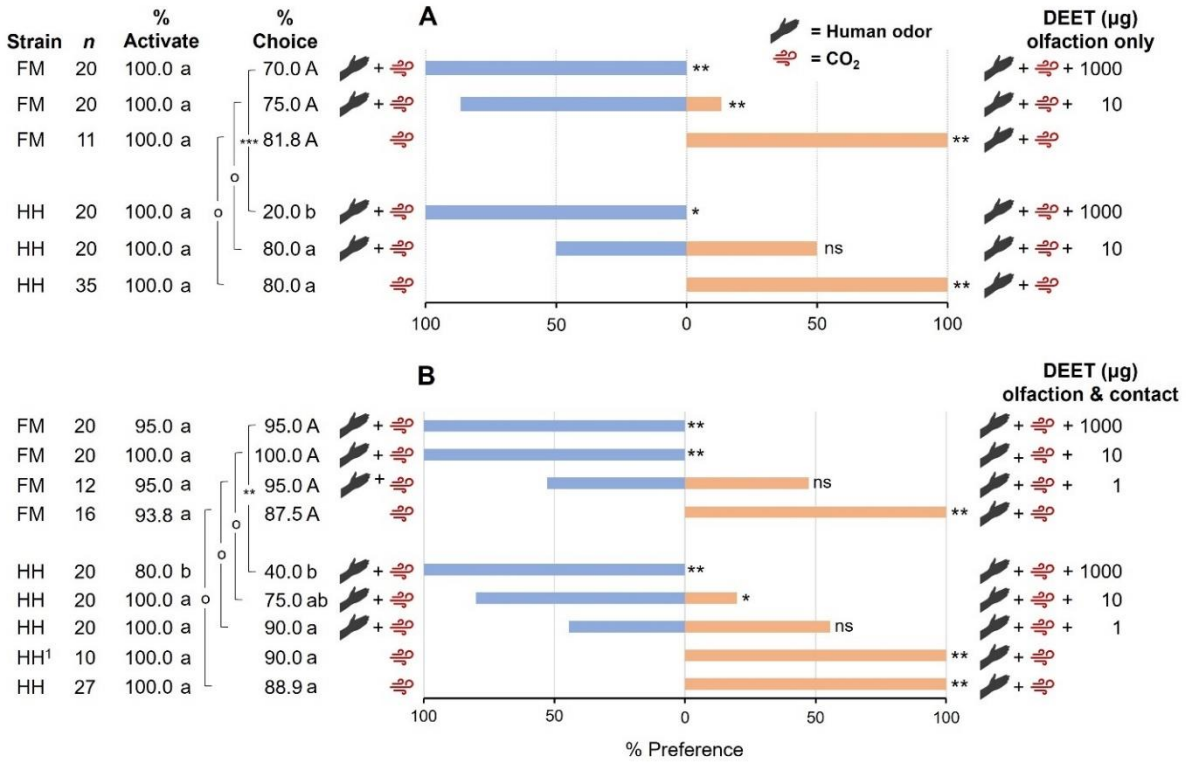

Fig. 3.3. Comparisons of (**A**) olfaction-mediated and (**B**) olfaction- plus contact-mediated behavioral responses of insecticide susceptible (HH strain) and insecticide resistant (FM strain) *C. lectularius* to various doses of DEET. Individual 10–14 d starved females of both strains were provided host-associated stimuli, namely human odor and/or CO_2. The positive control (bottom treatment group in each graph) consisted of human odor and CO_2 at one arm of the olfactometer and CO_2-only at the other arm. A subset of HH (HH^1) were run as method validations to ensure that the addition of paper walkways did not significantly alter behavior. The percentages of activated bed bugs that chose either arm of the olfactometer are shown (% choice). The aggregate percentage preference toward the subject stimuli, including DEET (orange), and towards the control stimuli (blue) are shown. Percentages activation, choice, and preference were compared separately based on exposure modality using identical methods. Activation rates were compared across strains, and those that share lower case letters are not significantly different (GLM, Tukey's HSD, $P < 0.05$). Percentages making a Choice were compared within strains (One-way ANOVA, Tukey's HSD) and between strains (GLM, Tukey's HSD), and those that share case-specific letters are not significantly different. Between strain comparisons are denoted by brackets, with significance denoted by: o, ns; **, $P < 0.01$; and ***, $P < 0.001$. Preference was compared independently at each dose by Chi-square test, with asterisks representing significant differences in preference denoted by *, $P < 0.05$; and **, $P < 0.01$.

Table 3.1. Comparison of average latency to activation (s) across strains, doses of DEET, and exposure modalities.

Treatment	**Figure 2**			**Figure 3A**			**Figure 3B**		
	No CO_2	**CO_2**	***P*-value[c]**	**HH**	**FM**	***P*-value[c]**	**HH**	**FM**	***P*-value[c]**
Positive control[a]	87.2	57.3	0.0763	59.4	94.9	0.0953	52.8	99.5	0.0065
Side-bias control[b]	62.0	37.6	0.1089	-	-	-	17.8	-	0.0023[d]
DEET (μg)									
0.01	65.8	53.6	0.2864	-	-	-	-	-	-
1	45.6	27.8	0.1446	-	-	-	64.7	56.3	0.3261
10	84.5	48.0	0.0719	48.0	78.4	0.1229	32.2	44.2	0.1653
100	67.1	58.1	0.3297	-	-	-	-	-	-
1000	70.8	75.7	0.4484	75.7	55.3	0.2182	46.7	35.3	0.2815

[a]Assays run with attractive host cues (human skin swab without (Fig. 2A) or with CO_2 (Fig. 2B) in one arm and either air only (Fig. 2A) or CO_2 only (Fig. 2B) in the other arm of the olfactometer.

[b]Assays run with attractive host cues in both arms of the olfactometer to assess for potential side-bias of the olfactometer.

[c]Comparisons performed via individual Student's *t*-tests

[d]Compared to HH positive control

Table 3.2. Comparison of average latency to making a choice (s) across strains, doses of DEET, and exposure modalities.

Treatment	**Figure 2**			**Figure 3A**			**Figure 3B**		
	No CO_2	**CO_2**	***P*-value[c]**	**HH**	**FM**	***P*-value[c]**	**HH**	**FM**	***P*-value[c]**
Positive control[a]	224.3	190.6	0.1116	203.9	227.7	0.2005	99.5	148	0.0321
Side-bias control[b]	222.5	132.5	0.0033	-	-	-	70.1	-	0.1043^
DEET (µg)									
0.01	251.5	200.9	0.0817	-	-	-	-	-	-
1	251.4	167.9	0.0044	-	-	-	147.2	92.2	0.0229
10	260.3	218.0	0.1855	218.0	195.7	0.2652	77.7	96.5	0.1829
100	213.3	171.9	0.2461	-	-	-	-	-	-
1000	255.0	189.5	0.2989	189.5	166.9	0.3674	76.0	65.1	0.3245

[a]Assays run with attractive host cues (human skin swabs with/without CO_2) in one arm and either air (CO_2 assays) or CO_2 alone (assays involving both strains) in the other arm.
[b]Assays run with attractive host cues in both arms to assess effects of assay design on bed bug latency to choice (s). Namely, sidedness (No CO_2 vs. CO_2) and modifying the walking surface (olfaction + contact assays).
[c]Comparisons performed via individual t-tests
[d]Compared to HH positive control

CHAPTER 4

Repellency and toxicity of commonly used long-lasting insecticide-treated bed nets (LLINs) to bed bugs

(This work is being prepared for submission to the *Journal of Medical Entomology*: **Hayes CC**, and **Schal C.** 2024 Repellency and toxicity of commonly used long-lasting insecticide-treated bed nets (LLINs) to bed bugs. *Journal of Medical Entomology*. (in-prep).)

Abstract

Vector control is essential for eliminating malaria, a vector-borne parasitic disease responsible for over half a million deaths annually, and program success hinges on community acceptance of products like long-lasting insecticide-treated nets (LLINs). Communities in malaria-endemic regions often link LLIN efficacy to their ability to control indoor pest populations such as bed bugs (*Cimex lectularius* L. and *C. hemipterus* F.) (Hemiptera: Cimicidae). Despite this, little is known about the potential repellent effects and toxicity of LLINs to bed bugs. Herein, we quantify for the first time that commonly deployed LLINs lack olfactory and contact-based repellency to host-seeking insecticide susceptible and insecticide resistant *C. lectularius* bed bugs. One LLIN (PermaNet Dual) was significantly attractive to both strains when exposed olfactorily, but not in contact assays, highlighting the complexity of bed bug-LLIN interactions. The insecticide resistant bed bugs experienced low mortality in 96 h continuous exposure to LLINs. Our results suggest that LLINs would likely not repel or eliminate bed bug infestations in malaria-endemic communities, further selecting for insecticide resistance and potentially disrupting vector control programs.

Introduction

Malaria, a parasitic disease caused by infection with *Plasmodium* (Apicomplexa: Plasmodiidae) and vectored to humans through the bite of infected *Anopheles* mosquitoes (Diptera: Culicidae), is responsible for nearly half a million deaths annually, mainly in sub-Saharan Africa (World Health Organization 2022). This immense burden makes malaria control and elimination a global priority (Feachem et al. 2010). To date, the majority of averted disease has been achieved by intervention efforts in two broad areas: 1) treatment of the disease, and 2) disruption of contact between the mosquito vector and potential hosts (Shretta et al. 2017, Mendis 2019, Wilson et al. 2020). The use of Long-Lasting Insecticide-treated Nets (LLINs) has been the single most effective malaria prevention strategy to-date (Bhatt et al. 2015). However, the use of indoor LLINs places them in the habitat of domiciliary pests, such as the bed bugs *Cimex lectularius* L. and *Cimex hemipterus* (F.) (Hemiptera: Cimicidae)(Hayes and Schal 2024a). Both species have resurged globally in the past two decades in large part due to the widespread emergence of insecticide resistance (Dang et al. 2017, Lewis et al. 2023) and have become prolific, persistent, and difficult to control pests.

Malaria-endemic communities that consider the use of LLINs often prioritize the immediate benefits of indoor pest control over the long-term health benefits of mitigating malaria transmission (Helitzer et al. 1993, Temu et al. 1999, Sharma et al. 2009). Thus, failure to control indoor pests can contribute to LLIN abandonment, misuse, and ultimately lower efficacy of indoor vector control programs (Ingabire et al. 2015, Malede et al. 2019). In a recent review of these interactions (Hayes and Schal 2024a), we highlighted that LLINs can impose strong selection pressure on bed bugs because of their low mobility, tendency to bite their sleeping human host at night, and high affinity for aggregation sites near but not on the host (Johnson

1941, Olson et al. 2017, Gaire et al. 2021). These behaviors place bed bugs in frequent contact with deployed LLINs (Hayes and Schal 2022).

Few studies have reported on the behavioral and ecological interactions of bed bugs with insecticide treated bed nets. Recently, we showed that multiple life stages of insecticide-susceptible and insecticide-resistant *C. lectularius* bed bugs were able to pass through pyrethroid-treated LLINs both in pursuit of a host and when returning to aggregation sites, with minimal mortality only in the insecticide-susceptible bed bugs due to brief interactions with the nets (Hayes and Schal 2022). In these assays we saw no evidence of LLIN repellency of bed bugs. Because these were largely end-point assays that did not track behavior, it remains unknown whether LLINs repel bed bugs. Therefore, we validated the use of a two-choice olfactometer (DeVries et al. 2019) for repellency assays, and then demonstrated its use for the analysis of repellency in bed bugs using the "gold standard" repellent, DEET (Hayes and Schal 2024b). Using this bioassay, we evaluated the olfactory and olfactory plus contact repellency of commonly used LLINs, as well as bed bug mortality associated with continuousexposure. This work was conducted with a highly insecticide-susceptible reference strain and a highly insecticide-resistant strain to explore the possibility that resistance to insecticides might alter the chemosensory sensitivity of bed bugs to LLINs.

Materials and Methods

Colony Maintenance and Feeding

Two strains of *C. lectularius* were used in this study. The Harold Harlan strain (HH, also known as Ft. Dix) is a commonly used insecticide susceptible reference strain collected at Fort Dix, New Jersey (USA) in 1973 and has not been challenged with insecticides since collection. It was

maintained on a human host until December 2008, and then, in our lab, on defibrinated rabbit blood until July 2021 and on human blood thereafter. The Fuller Mill Road strain (FM) is resistant to multiple classes of insecticides and was collected from a residence in High Point, North Carolina (USA) in 2017, maintained in our lab on defibrinated rabbit blood until July 2021 and on human blood thereafter (González-Morales et al. 2022). Both strains were maintained at 35–45% relative humidity, 25ºC on a 12:12 (L:D) h photoperiod and fed weekly on heparinized human blood (supplied by the American Red Cross under IRB #00000288 and protocol #2018-026) using a previously described feeding system (Sierras and Schal 2017, Hayes and Schal 2022).

Only adult females were used in all repellency assays due to their need to obtain a blood meal between each oviposition cycle and thus high motivation to orient towards human odor. Groups of 20–30 females were separated from colony jars within 48 h post-feeding and starved for 10–14 days at 35–45% relative humidity, 25ºC on a 12:12 (L:D) h photoperiod. Since the females were of unknown ages and likely mated within the colony, at several points during this period, but not within 24 h of the assay, groups of 20-30 females were moved onto clean folder paper in clean 20 ml clear scintillation vials (DWK Life Sciences, Millville, NJ) to remove all eggs. Each bed bug was used for a single bioassay, then discarded. Adult male bed bugs were used in survival assays 4–5 days after they blood-fed.

Human Odor Preparation

Human odor was collected from the primary researcher (CCH) following a previously validated and approved SOP for human skin swab collection (NCSU IRB Protocol 14173). Briefly, no alcohol, spicy, or pungent foods were consumed for at least 24 h prior to collection.

Approximately 2–10 h before odor collection CCH showered using Cetaphil ultra gentle body wash (Galderma, Fort Worth, TX) and no shampoo. No deodorant was applied, and no strenuous activities performed. Hourly, between 2–10 h after showering, CCH cleansed his hands with water (no soap) and once dried, a filter paper (#1, 90 mm diameter, Whatman, Maidstone, United Kingdom) was used to swab a single forearm from wrist to elbow, armpit, and leg from ankle to knee for 30 s per side. A second filter paper was used to swab the same regions on the other side of his body. Each filter paper was cut into 16 equal pie-shaped pieces (4 cm^2 each) stored in a glass vial at -20°C and used within one month.

Bed Net Sample Preparation

We used 5 different bed nets, including 4 different LLINs and an untreated control bed net. These were (1) Siam Dutch (SD), an untreated bed net (Siam Dutch Mosquito Netting Co., Bangkok, Thailand); (2) Olyset Net (OY), containing 800 mg permethrin/m^2 (Sumitomo Chemical, Osaka, Japan); (3) PermaNet 2.0 (PN 2.0), containing 56 mg deltamethrin/m^2 (Vestergaard, Lausanne, Switzerland); (4) PermaNet 3.0 (PN 3.0), containing two distinct treatments (side (S) panel and roof (R) panel) which were assessed separately and are henceforth referred to as PermaNet 3.0S (PN 3.0S) which contains 84 mg deltamethrin/m^2, and PermaNet 3.0R (PN 3.0R) which contains 120 mg deltamethrin/m^2 plus 800 mg piperonyl butoxide (PBO)/m^2 (Vestergaard); and (5) PermaNet Dual (PND), containing 84 mg deltamethrin/m^2 plus 200 mg chlorfenapyr/m^2 (Vestergaard). The SD and OY nets were provided as new nets by the US Centers for Disease Control (CDC, Atlanta, GA). Cuttings of newly manufactured PermaNets from two distinct production runs were provided by Vestergaard. Individual squares (15 cm x 15 cm; 0.0225 m^2) of each bed net were prepared for olfaction-only repellency assays,

and rectangles (1.5 cm x 3.0 cm; 0.00045 m^2) for olfaction plus contact assays. Squares used in olfaction assays were replaced weekly, and rectangles used in olfaction plus contact assays were replaced between each assay. Individual squares (8 cm x 8 cm; 0.0064 m^2) of each bed net were prepared for survival assays, for each replicate, and then discarded. In all manipulations of bed nets, chemicals, olfactometer components, and bed bugs, Layer4® nitrile gloves (USA Scientific, Ocala, FL) were worn, and they were replaced regularly to prevent cross-contamination and transfer of human skin compounds.

Chlorfenapyr Dispenser Preparation

Individual squares (1.5 cm x 1.5 cm; 0.000225 m^2) of #1 Whatman filter papers were placed on an aluminum foil, treated with 10 µl of either 99.9% acetone (Sigma-Aldrich, Burlington, MA) (control) or 98.0% chlorfenapyr (PESTANAL analytical standard, Fisher Scientific, Waltham, MA) in acetone, allowed to air dry for at least 20 min, and stored in glass vials (20 ml) at -20°C. Concentrations of chlorfenapyr (PESTANAL analytical standard, Fisher Scientific, Waltham, MA) tested were 0.1, 1.0, and 10.0 µg/µl, representing total doses of 1, 10, and 100 µg, respectively, applied to each filter paper.

Olfactometer Assays

All assays were performed using a glass Y-tube olfactometer and methodology as previously described (Hayes and Schal 2024b) , with minor modifications. Briefly, a vertically oriented Y-tube was connected to a forced air system (**Fig. 4.1**). A plankton mesh (Wildco, Yulee, FL) walkway was used to facilitate bed bugs crawling on a vertical surface, and it was replaced after no more than five replicate assays or for each assayed bed net, whichever came first.

In olfaction-only LLIN assays (**Fig. 4.1A**), medical quality humidified air (200 ml/min) (Airgas Healthcare, Radnor, PA) was split so 100 ml/min passed through each glass jar (8 cm x 12.5 cm; ~628.3 cm^3) (Prism Glass, Raleigh, NC) that contained a square cutting of a bed net, rotating between samples from different production runs when possible (PermaNet samples), each day of data collection. A square cutting of an untreated bed net (SD) was placed in one of the bed net jars and one of the four LLINs was placed in the other. Then, CO_2 (0.6 ml/min, ~3,000 ppm, Airgas Healthcare, Radnor, PA) was added to each air stream before it entered the olfactometer. At the distal end of each arm of the olfactometer we placed a human skin swab. In olfaction plus contact LLIN assays (**Fig. 4.1B**), all parameters were identical to the olfaction-only assay, except that odor jars containing LLIN cuttings were removed, and a rectangular cutting of bed net was affixed to the walkway at the proximal end of one arm of the olfactometer, rotating between samples from different production runs for all PermaNet samples each day of data collection. A cutting of the untreated SD bed net was affixed to the walkway of the other arm. In the olfaction-only assays with chlorfenapyr, all parameters were identical to the LLIN olfaction assays, except that the LLIN jars shown in **Fig. 4.1A** were removed. A chlorfenapyr-treated filter paper, rather than LLIN cutting, was placed at the distal end of one arm of the olfactometer and an acetone-treated filter paper was placed in the other arm, each adjacent to a skin swab filter paper, but preventing contact of the two treated papers (**Fig. 4.1C**).

In all assays, individual bed bugs were acclimatized to 200 ml/min of air for at least 30 min in separate releasing tubes, and then introduced to the assay via the uncapped releasing tube. Activation (moving from the releasing tube into the common arm of the olfactometer), Choice (moving more than halfway up either of the assay arms (**Fig. 4.1A, C**) or crossing onto either the

untreated or treated bed net affixed to the walkway (**Fig. 4.1B**)), and Preference (selected assay arm) were recorded up to 5 min or to when a choice was made, whichever came first.

Mortality Assays

All mortality assays were performed in inverted plastic jars (5.5 cm × 4.8 cm each; Olcott Plastics, Saint Charles, IL) with the bottom of each jar, and the center of each lid, removed. Square cuttings of each bed net were affixed to the top of each jar and secured using the ring-shaped lid. Jars were then inverted, and groups of 10 adult male bed bugs were placed on each bed net, for both HH and FM strains, with all LLINs run in triplicate (n = 30 bed bugs per LLIN per strain). Bed bugs remained in continuous contact with the bed net surface throughout the assay, and mortality was scored at 0.25, 0.5, 1, 2, 3, 6, 8, 12 h and then every 12 h to 96 h. Bed bugs were considered dead if they failed to move when gently touched using feather light forceps and they were subsequently unable to right themselves when flipped onto their dorsal side.

Statistical Analysis

All statistical analyses were conducted using SAS Enterprise Guide (v. 8.3, SAS Institute, Cary, NC), with $\alpha = 0.05$. Percentage activation and choice data were first arcsine square-root-transformed, and then compared via one-way ANOVA within strain, and generalized linear model (GLM) followed by Tukey's HSD between strains. Within strain LLIN-specific percentage preference was compared using individual Chi-square tests. Comparison of proportion surviving on each LLIN over time was done using Kaplan-Meier survival analysis and log-rank tests with Sidak correction for multiple comparisons.

Results

Lack of Olfactory Repellency of LLINs

Using the olfaction-only bed net assay (**Fig. 4.1A**) we evaluated the repellency of five bed net treatments representing four commonly distributed LLINs, as well as an untreated bed net control. High percentage activation (91.7%) and choice (86.4%) were seen with the HH bed bugs in the positive controls (human odor and CO_2 only in one arm) (**Fig. 4.2A**). Similarly, in assays run with the FM strain, both positive controls and concurrently run HH strain controls (HC: run daily alongside FM controls to allow for cross-strain comparisons) showed high activation (95.2%, 91.3%) and choice (90.0%, 85.0%), respectively (**Fig. 4.2B**). In the HH, FM, and HC controls, 100% of the bugs that made a choice preferred the human odor-containing arm of the Y-tube (Chi-square test, HH: $\chi^2 = 19$, $df = 1$, $P < 0.01$; FM: $\chi^2 = 18$, $df = 1$, $P < 0.01$; HC: $\chi^2 = 18$, $df = 1$, $P < 0.01$). We validated the untreated SD net as a negative control (NC) by placing a square cutting on one side of the olfactometer with human odor and CO_2 supplied on both sides, revealing similarly high percent activation (100%), percent choice (77.8%), and no significant preference for the arm containing the SD net ($\chi^2 = 0.3$, $df = 1$, $P > 0.05$).

The HH bed bugs showed overall high percentage activation across all LLIN assays (**Fig. 4.2A**) (80.0–95.0%), with no significant change in activation across tested LLINs (one-way ANOVA, $F = 1.12$, $df = 6,27$, $P = 0.3758$). Similarly, 85.0–100% activation was seen across all LLIN assays with the FM strain bed bugs (**Fig. 4.2B**), and no significant change in activation across LLINs (one-way ANOVA, $F = 1.17$, $df = 5,23$, $P = 0.3692$). Comparison of activation between strains revealed an overall insignificant model (GLM, $F = 1.14$, $df = 12,59$, $P = 0.3505$) with neither strain ($F = 3.61$, $df = 1$, $P = 0.0622$), LLIN ($F = 1.38$, $df = 6$, $P = 0.2388$), or the interaction of strain and LLIN ($F = 0.89$, $df = 5$, $P = 0.4924$) significantly affecting percentage

activation. Percentage choice was also high in both HH (64.7–89.5%) (**Fig. 4.2A**) and FM (82.4–95.0%) bed bugs (**Fig. 4.2B**), with no significant decrease in choice associated with LLIN in either strain (one-way ANOVA, HH: $F = 0.87$, $df = 6,27$, $P = 0.5279$; FM: $F = 0.69$, $df = 5,23$, $P = 0.6355$). Comparison of percentage choice between strains revealed an overall insignificant model (GLM, $F = 1.17$, $df = 12,59$, $P = 0.3287$) with neither strain ($F = 3.87$, $df = 1$, $P = 0.0538$), treatment ($F = 1.51$, $df = 6$, $P = 0.1898$), or the interaction of strain and treatment ($F = 0.17$, $df = 5$, $P = 0.9719$) significantly affecting percentage choice.

Comparison of percent preference across treatments revealed unexpected results in both strains. In both HH and FM bed bugs we saw no significant attraction or repellency when exposed to any of the single-ingredient or synergist-containing LLINs (Chi-square tests, OY: HH – $\chi^2 = 0.0$, $df = 1$, $P > 0.05$, FM – $\chi^2 = 2.57$, $df = 1$, $P > 0.05$; PN 2.0: HH – $\chi^2 = 0.33$, $df = 1$, $P > 0.05$, FM – $\chi^2 = 3.6$, $df = 1$, $P > 0.05$; PN 3.0S: HH – $\chi^2 = 2.2$, $df = 1$, $P > 0.05$, FM – $\chi^2 = 0.53$, $df = 1$, $P > 0.05$; PN 3.0R: HH – $\chi^2 = 0.53$, $df = 1$, $P > 0.05$, FM – $\chi^2 = 0.47$, $df = 1$, $P > 0.05$) (**Fig. 4.2**). However, we saw surprisingly significant attraction to the chlorfenapyr-containing PND net in both the HH bed bugs ($\chi^2 = 13.24$, $df = 1$, $P < 0.01$), and to a lesser degree in FM bed bugs ($\chi^2 = 4.26$, $df = 1$, $P < 0.05$). These results indicate that while all LLINs failed to repel insecticide-susceptible and resistant bed bugs, PND attracted bed bugs.

Marginal Repellency of LLINs in Contact Assays

To further investigate the potential attractiveness of the PND LLIN, and to introduce contact with LLINs as an additional sensory modality, we assessed repellency in response to both olfactory and contact stimuli in the same representative bed nets (**Fig. 4.1B**). Once again, we saw 93.3% activation and 85.7% choice in the HH strain positive controls (**Fig. 4.3A**), as well as in

HH bed bugs concurrently run with FM bed bugs (HC controls, 100% and 90.9%, respectively) (**Fig. 4.3B**). All FM positive control bed bugs activated and made a choice (**Fig. 4.3B**). In all HH, FM, and HC controls, 100% of bed bugs that made a choice preferred the human odor-containing arm of the Y-tube (Chi-square test, HH: $\chi^2 = 12$, $df = 1$, $P < 0.01$; FM: $\chi^2 = 10$, $df = 1$, $P < 0.01$; HC: $\chi^2 = 10$, $df = 1$, $P < 0.01$).

The olfaction plus contact assays also resulted in high overall activation in both the HH (85.0–100%) and the FM (95.0–100%) bed bugs, with no significant decrease in activation based on LLIN for either strain (one-way ANOVA, HH: $F = 0.89$, $df = 5,20$, $P = 0.5039$; FM: $F = 0.46$, $df = 5,19$, $P = 0.8038$). Comparison of percentage activation across both strains revealed an overall insignificant model (GLM, $F = 0.76$, $df = 11,44$, $P = 0.6740$), with neither strain ($F = 0.12$, $df = 1$, $P = 0.7339$), LLIN ($F = 1.22$, $df = 5$, $P = 0.3156$), or the interaction of strain and LLIN ($F = 0.47$, $df = 5$, $P = 0.7948$) significantly affecting activation. Further, percentage choice remained high across both strains (HH: 60.0–85.0%; FM: 89.5–95.0%) regardless of LLIN, with no significant decrease in percentage choice associated with LLIN in either strain (one-way ANOVA, HH: $F = 2.11$, $df = 5,25$, $P = 0.0979$; FM: $F = 1.02$, $df = 5,19$, $P = 0.4361$) (**Fig. 4.3**). Comparison between strains of the percentage bed bugs that made a choice revealed an overall significant model (GLM, $F = 2.46$, $df = 11,44$, $P = 0.0170$) with strain significantly affecting choice ($F = 14.56$, $df = 1$, $P = 0.0004$), but neither LLIN ($F = 2.36$, $df = 5$, $P = 0.0557$) nor the interaction of strain and LLIN ($F = 0.53$, $df = 5$, $P = 0.7494$) significantly affecting percentage choice.

Comparison of percentage preference across treatments following the addition of contact exposure in the HH strain revealed no significant attraction or repellency when exposed to three of the four LLINs (OY: $\chi^2 = 1.3$, $df = 1$, $P > 0.05$; PN 2.0: $\chi^2 = 1.5$, $df = 1$, $P > 0.05$; PN 3.0S: χ^2

= 0.0, *df* = 1, *P* > 0.05). However, PN 3.0R was significantly repellent to the HH strain bed bugs (χ^2 = 5.4, *df* = 1, *P* < 0.05) (**Fig. 4.3A**). In the FM strain we saw no significant attraction or repellency when bed bugs were exposed to any of the single-ingredient or synergist-containing LLINs (OY: χ^2 = 0.06, *df* = 1, *P* > 0.05; PN 2.0: χ^2 = 1.5, *df* = 1, *P* > 0.05; PN 3.0S: χ^2 = 0.47, *df* = 1, *P* > 0.05; PN 3.0R: χ^2 = 2.9, *df* = 1, *P* > 0.05) (**Fig. 4.3B**). In both strains, the previously observed attraction to the PND LLIN was eliminated upon the addition of contact exposure (HH: χ^2 = 1.9, *df* = 1, *P* > 0.05; FM: χ^2 = 0.47, *df* = 1, *P* > 0.05). Thus, both single- and multi-AI impregnated LLINs (except for PN 3.0R and HH strain) did not significantly repel host-seeking bed bugs.

No Olfactory Attraction or Repellency to Chlorfenapyr

We sought to understand why the PND bed net was attractive to bed bugs. Because other deltamethrin-containing LLINs (PN 2.0, 3.0S, 3.0R) did not attract bed bugs, we tested whether chlorfenapyr might attract bed bugs (**Fig. 4.1C**). In the presence of human odor and CO_2 100% of the bed bugs were activated and 90.0% made a choice (χ^2 = 11, *df* = 1, *P* < 0.01) (**Fig. 4.4**). Also, in the presence of technical chlorfenapyr, 85.0–95.0% of the bed bugs activated with no significant differences across tested doses of 1, 10 and 100 µg (one-way ANOVA, *F* = 2.11, *df* = 3,12, *P* = 0.1522). Similarly, high percentages of the bed bugs made a choice across all tested doses (76.5–84.2%) with no significant effect of chlorfenapyr dose (one-way ANOVA, *F* = 0.62, *df* = 3,12, *P* = 0.6176). Finally, there was no significant preference across doses of chlorfenapyr (1 µg: χ^2 = 0.5, *df* = 1, *P* > 0.05; 10 µg: χ^2 = 1.8, *df* = 1, *P* > 0.05; 100 µg: χ^2 = 3.77, *df* = 1, *P* > 0.05), suggesting no significant repellency of chlorfenapyr. However, a trend across the three doses suggests that higher doses of chlorfenapyr might repel HH bed bugs (**Fig. 4.4**).

Bed Bug Mortality on LLINs

Because the LLINs were not repellent to both bed bug strains in olfactory (**Fig. 4.2**) and olfactory plus contact assays (**Fig. 4.3**), we surmised that bed bugs might frequently contact the LLINs and possibly harbor in the creases of the LLINs under field conditions. Therefore, we exposed 180 bed bugs of each strain to five LLINs treatments in 4-day long continuous contact assays. The HH bed bugs reached 100% mortality across all LLINs within 12 h, whereas 100% of the bed bugs survived on the SD untreated bed nets for 96 h (shown to 16 h in **Fig. 4.5A**). The resistant FM bed bugs reached only 80% mortality in a single LLIN treatment (PN 3.0R: deltamethrin + PBO) by 96 h, with 100% survival on the SD controls (**Fig. 4.5B**). Comparison of strain-specific survival revealed significantly lower proportion surviving on LLINs in both the HH (Kaplan-Meier, log-rank, $\chi^2 = 261.9$, $df = 1$, $P < 0.0001$) and FM ($\chi^2 = 46.0$, $df = 1$, $P < 0.0001$) strains (**Fig. 4.5**) compared to the untreated SD control. Comparison of the two strains by LLIN revealed that significantly higher proportions of FM bed bugs than HH bed bugs survived on all the LLINs. Analysis of survival over-time revealed overall similar survival time in both HH and FM bed bugs regardless of LLIN, with PN 3.0R causing the most rapid mortality (log-rank, HH: $\chi^2 = 175.8$, $df = 1$, $P < 0.0001$; FM: $\chi^2 = 41.9$, $df = 1$, $P < 0.0001$). These results demonstrate that with long uninterrupted LLIN exposure representing sheltering by blood-fed bed bugs, only a fraction of the insecticide-resistant bed bugs (FM strain) died (20–67% survival), suggesting that LLINs can impose strong selection pressure for the emergence and maintenance of insecticide resistance in bed bug populations.

Discussion

To our knowledge, this is the first study to 1) compare the olfactory and contact repellency of commonly used LLINs to both insecticide-susceptible and insecticide-resistant bed bugs, 2)

assess the survival of bed bugs over time with continuous exposure to multiple LLINs, and unexpectedly 3) present empirical data showing behavioral olfactory attraction of bed bugs to an insecticide-impregnated fabric, and loss of attraction following the addition of contact exposure. We were able to show this through the use of a robust binary choice olfactometer system that was previously validated to assess repellency in bed bugs (Hayes and Schal 2024b). Briefly, we have shown that olfactory exposure to commonly distributed single- and multi-ingredient LLINs did not repel either insecticide-susceptible or insecticide-resistant *C. lectularius* (**Fig. 4.2**). Further, the addition of contact exposure to the same LLINs also did not repel bed bugs of both strains, with the exception of the HH strain bed bugs exposed to PN 3.0R (deltamethrin + PBO) (**Fig. 4.3A**).

Repellency of Insecticides to Bed Bugs

Our understanding of insecticide repellency to bed bugs is lacking, and even more so as it relates to the association between insecticide resistance and changes in repellency. Different insecticide resistant bed bug strains appear to exhibit differing responses to DEET, some showing lower and others expressing higher sensitivity to DEET (Vassena et al. 2019, Hayes and Schal 2024b), consistent with findings in other insect species (Wagman et al. 2015, Deletre et al. 2019, Fardisi et al. 2019, Andreazza et al. 2021). Of particular interest is repellency of bed bugs to pyrethroids, the most widely used class of insecticides. Here too, both repellency and lack of repellency have been reported for the same pyrethroid insecticides (Moore and Miller 2006, Romero et al. 2009a, Jones et al. 2013, Wang et al. 2013). Notably, different repellency assays might account for disparate results, including the use of olfactory- or contact-based assays and observational vs. endpoint assays.

Herein, we have provided clear evidence that LLINs containing pyrethroids, irrespective of exposure modality, generally do not repel host-seeking, insecticide-susceptible and insecticide-resistant bed bugs (**Figs. 4.2, 4.3**). Nonetheless, we observed significant repellency of HH strain bed bugs in olfaction plus contact assays with PN 3.0R, which contains 120 mg/m^2 deltamethrin plus 800 mg/m^2 PBO. It is unclear however what aspect of this LLIN is repellent to bed bugs. Also, PN 3.0R is the roof panel of the PermaNet 3.0 LLIN, and the likelihood of frequent bed bug contact with the LLIN roof during host-seeking in the field is not known. Previous research has demonstrated the clear synergistic effects of PBO when used in concert with pyrethroids, both in bed bugs and other insect species (Romero et al. 2009b, How and Lee 2011, Gonzalez-Morales and Romero 2018, Sahu et al. 2019). Despite its utility as a synergist in overcoming insecticide resistance, it has been shown that the application of PBO within vector control settings can disrupt applied residual insecticides, highlighting its complex nature (Gleave et al. 2021, Syme et al. 2022). Importantly, PBO elicits irritancy behaviors in field populations of the mosquito *Aedes aegypti* (L.) (Diptera: Culicidae) (Yu et al. 2021), suggesting that PBO in PermaNet 3.0 LLIN might be responsible for contact repellency in bed bugs. However, this mechanism might be limited to pyrethroid-susceptible bed bugs, which are rarely found in recent global collections (Dang et al. 2017). The increasing reliance on PBO and other synergists to overcome pyrethroid resistance in bed bugs, and its use in LLINs and indoor residual sprays compel more research on its contact repellency to bed bugs and disease vectors as it might lessen their intended contact with applied products.

Attraction of Bed Bugs to PermaNet Dual

We demonstrated attraction of *C. lectularius* to PermaNet Dual, a dual-AI LLIN with 84 mg/m^2 deltamethrin and 200 mg/m^2 chlorfenapyr (approximately 4.5 mg of chlorfenapyr in the 0.0225 m^2 LLIN cutting used in olfaction assays) (**Fig. 4.2**). We note that this finding represented two production lots of this bed net. To our knowledge, this is the first documented case of olfactory attraction of an insect to an insecticide-impregnated fabric. However, bed bugs were not attracted to three other tested LLINs that contained deltamethrin (PermaNet 2.0, 56 mg/m^2 deltamethrin; the side panel of PermaNet 3.0 (PN 3.0S), 84 mg/m^2 deltamethrin; and the roof of PermaNet 3.0 (PN 3.0R), 120 mg/m^2 deltamethrin and 800 mg/m^2 PBO) (**Fig. 4.3**), nor to various concentrations of technical grade chlorfenapyr (**Fig. 4.4**). Therefore, we suspect that neither deltamethrin nor chlorfenapyr contributed to bed bug attraction. However, little is known about the interactions of bed bugs with technical chlorfenapyr, as most of the research with this AI has focused on the efficacy of formulated products (Romero et al. 2010, Choe and Campbell 2014, Ashbrook et al. 2017, Kells and Kells 2022). Therefore, it would be instructive to conduct more extensive behavioral assays with a wider range of chlorfenapyr concentrations. Two other possibilities might account for the observed attraction to PermaNet Dual. First, this LLIN might have been contaminated with attractive odors. This is unlikely however because other LLINs were also packaged by the same individual at Vestergaard, and no attraction was observed with those LLINs. The second possibility is that constituents on this LLIN (possibly chemicals used in production) contributed to bed bug attraction. This too is unlikely because both PermaNet Dual and PermaNet 3.0 are woven using the same base fabric and impregnated via the same process (Vestergaard, personal communication). It is essential to first independently confirm our finding

of attraction of bed bugs to PermaNet Dual, then elucidate the mechanisms with headspace analyses coupled with behavioral assays.

The phenomenon of LLINs attracting pest insects presents potential challenges and opportunities in pest management. Namely, when disease vectors, such as *Anopheles* mosquitoes, are found indoors, it might be advantageous to attract them to LLINs to facilitate contact with the insecticide and potentially kill the mosquitoes. However, in the case of flightless and obligatory domiciliary pests like bed bugs, attractive LLINs could move them closer to human hosts and more persistent contact with the LLIN might select more rapidly for insecticide resistance.

Bed Bug Survivorship on LLINs and Vector Control

We have performed the first comprehensive time-course of mortality of insecticide-susceptible and insecticide-resistant bed bugs exposed to a variety of LLINs representing field-deployed historic and modern products (**Fig. 4.5**). Our results demonstrate that while 100% of the insecticide-susceptible HH bed bugs died within 12 h, at most 80% of the insecticide-resistant FM bed bugs died after 96 h of continuous exposure. These findings suggest that while these LLINs are highly effective on mosquitoes, their field efficacy on bed bugs is likely mediocre, highlighting the long-cited concerns of communities in malaria-endemic regions with bed bug infestations (Busvine 1958, Lines et al. 1987, Charlwood and Dagoro 1989, Ansari and Razdan 2001, Deku et al. 2021). The declining effectiveness of LLINs on bed bugs and other domiciliary pests, due in large part to the emergence of insecticide resistance, has been shown to decrease community trust and acceptance of LLINs, challenging the efficacy of indoor vector control programs and even leading to program failure (Busvine and Pal 1969, Temu et al. 1999, Myamba

et al. 2002, Maxwell et al. 2006). In a recent review, we summarize these interactions and consider future directions (Hayes and Schal 2024a).

Conclusion

Long-lasting insecticide-treated bed nets have been the mainstay of malaria prevention for the past two decades and have contributed to dramatic reductions in the global malaria burden. However, despite the extensive use of LLINs in malaria-endemic communities, the demonstrated reliance of communities on LLINs for bed bug control, and the risks to indoor vector control programs in the wake of failed eradication of bed bug infestations, minimal research exists on the interactions of bed bugs, LLINs, and the user community. We have assessed – for the first time – the olfaction-based and contact-based repellency, as well as contact-associated mortality over time, of four commonly used LLINs using both insecticide-susceptible and insecticide-resistant strains of the bed bug *C. lectularius*. We empirically demonstrated a near complete lack of both olfactory and contact LLIN repellency, and the failure of all tested LLINs to eliminate a field-representative insecticide-resistant strain of bed bugs even with constant exposure to the LLINs. Failure to eliminate bed bug populations, together with the impressive ability of small bed bug propagules to withstand the adverse effects of inbreeding and establish large indoor populations, highlight the immense selection pressure imposed on bed bug populations by the widespread use of LLINs. These findings also underscore the need for WHO, NGOs and LLIN manufacturers to consider the adverse effects of bed bug infestations on campaigns to reduce malaria, and conversely, the ancillary benefits of bed bug elimination on LLINs adoption, use, and retention.

Declarations

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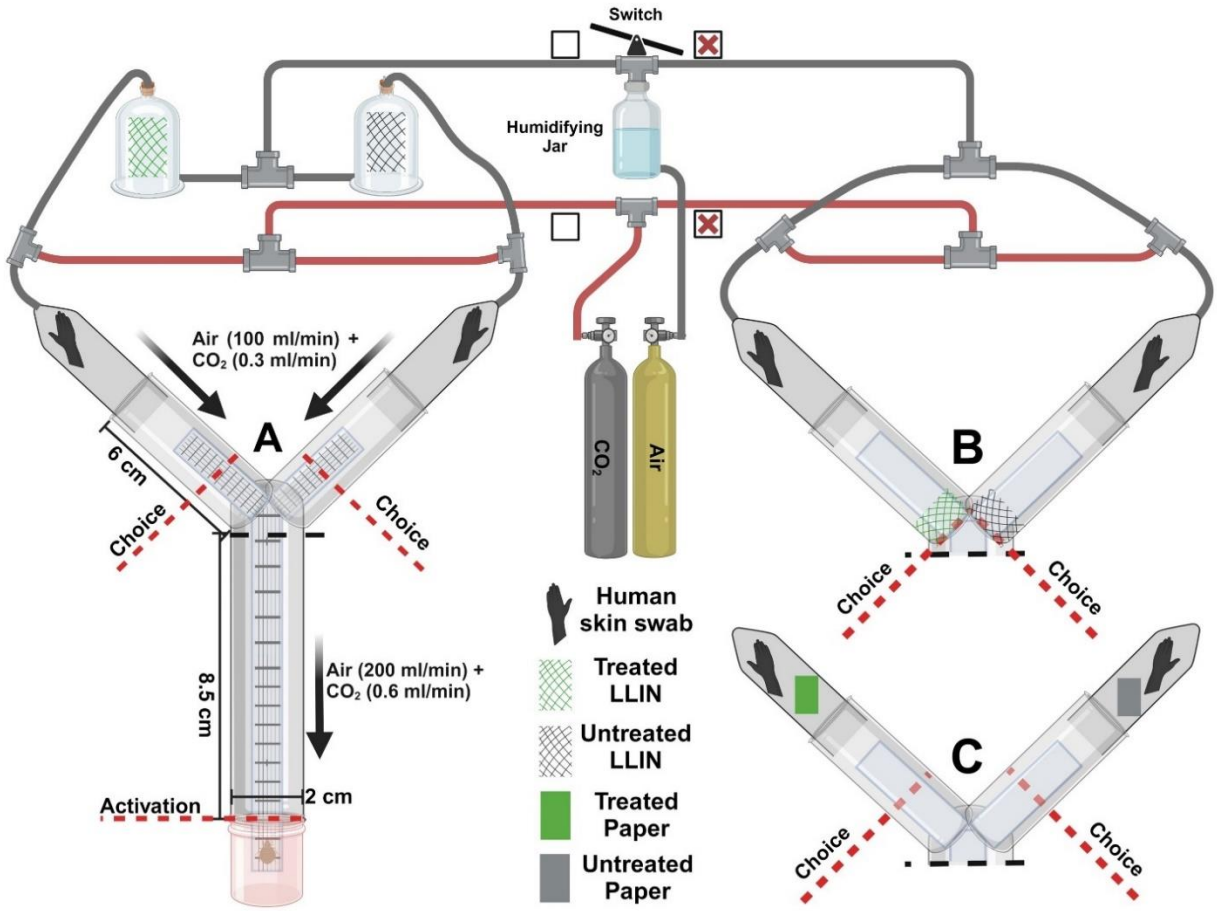

Fig. 4.1. Schematic of the binary choice olfactometer used to quantify the responses of *C. lectularius* to four different LLINs and to technical chlorfenapyr. Assays tested olfactory repellency without contact exposure (**A**, **C**) and the combination of olfactory and contact repellency (**B**). In all assays, host-seeking adult female bed bugs were attracted toward human-associated olfactory cues (skin volatiles and CO_2) delivered in humidified air. Glass jars containing cuttings of LLINs served to deliver LLIN odors in olfaction-only assays (**A**), but they were not used in olfaction plus contact assays (**B**) and chlorfenapyr olfaction assays (**C**). Behavioral responses to various LLINs and chlorfenapyr were quantified by measuring percentage Activation (bed bugs that entered the olfactometer / total bed bugs assayed); percentage Choice (bed bugs that made a choice of either arm of the olfactometer / total bed bugs that activated); and percentage Preference (percentage that chose the treatment vs. control arms of the olfactometer). LLINs, chlorfenapyr-treated filter papers, and human skin swab stimuli were prepared independently and introduced separately. Human skin swabs were replaced after each replicate, and the walkway was changed after either 5 replicates or between treatment groups, whichever came first. Created with BioRender.com.

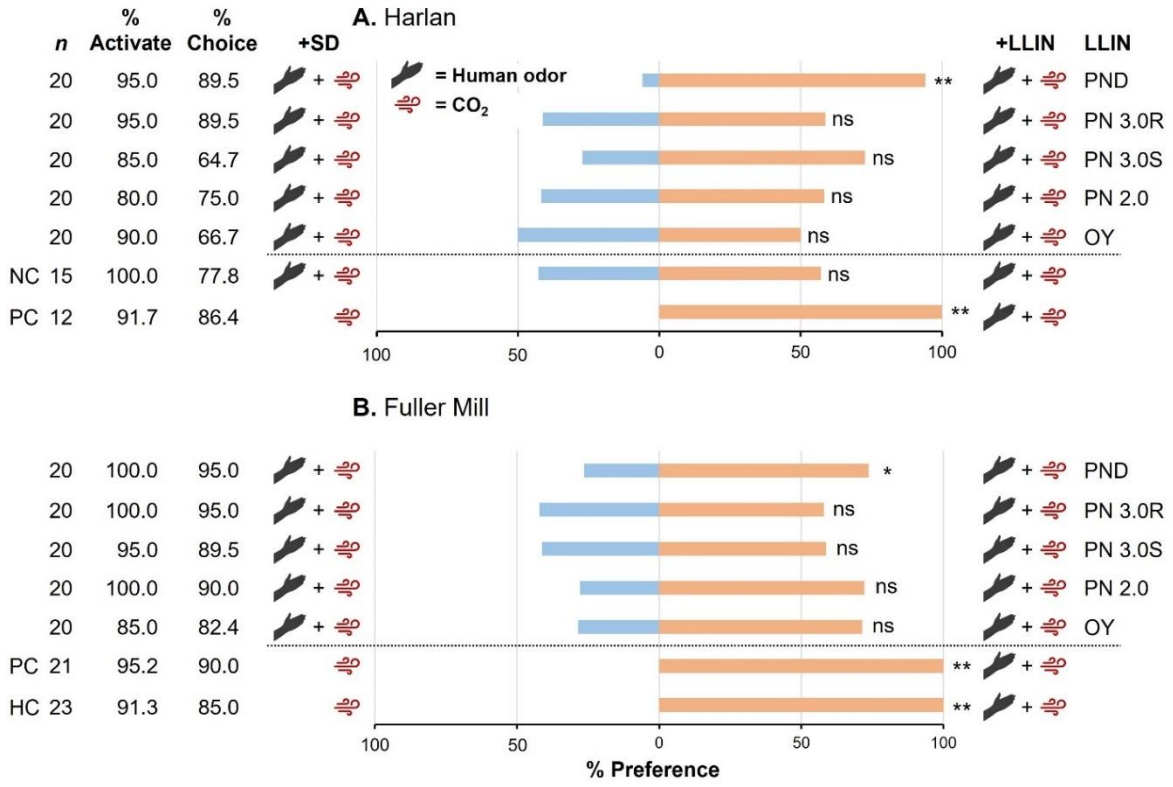

Fig. 4.2. Comparisons of olfaction-mediated behavioral responses of insecticide-susceptible (Harlan) (**A**) and insecticide-resistant (Fuller Mill) (**B**) *C. lectularius* to various LLINs. Individual adult females were assayed 10–14 d post blood meal. The positive control (PC) and the Harlan control (HC in **B**; run daily alongside Fuller Mill PC) consisted of host cues (human odor and/or CO_2) at only one arm of the olfactometer. The net negative control (NC in **A**) had identical host cues at both arms of the olfactometer. Activation was defined as the female leaving the release tube and entering the olfactometer, and % Activate is shown for each treatment group. The percentage of activated bed bugs that chose either arm of the olfactometer is shown as % Choice. The percentage choosing the subject stimuli, including LLIN (right side, orange), and towards the control stimuli, including the untreated Siam Dutch (SD) net (left side, blue) are shown. Percentage Activate was compared within each graph (one-way ANOVA) and the overall model for each was not significant ($P > 0.05$). Likewise, % Choice was compared within each graph (one-way ANOVA) and between strains (GLM), and both sets of comparisons did not reveal any significant differences ($P > 0.05$). Preference was compared independently for each LLIN by Chi-square test, with asterisks representing significant differences in preference denoted by *, $P < 0.05$; and **, $P < 0.01$.

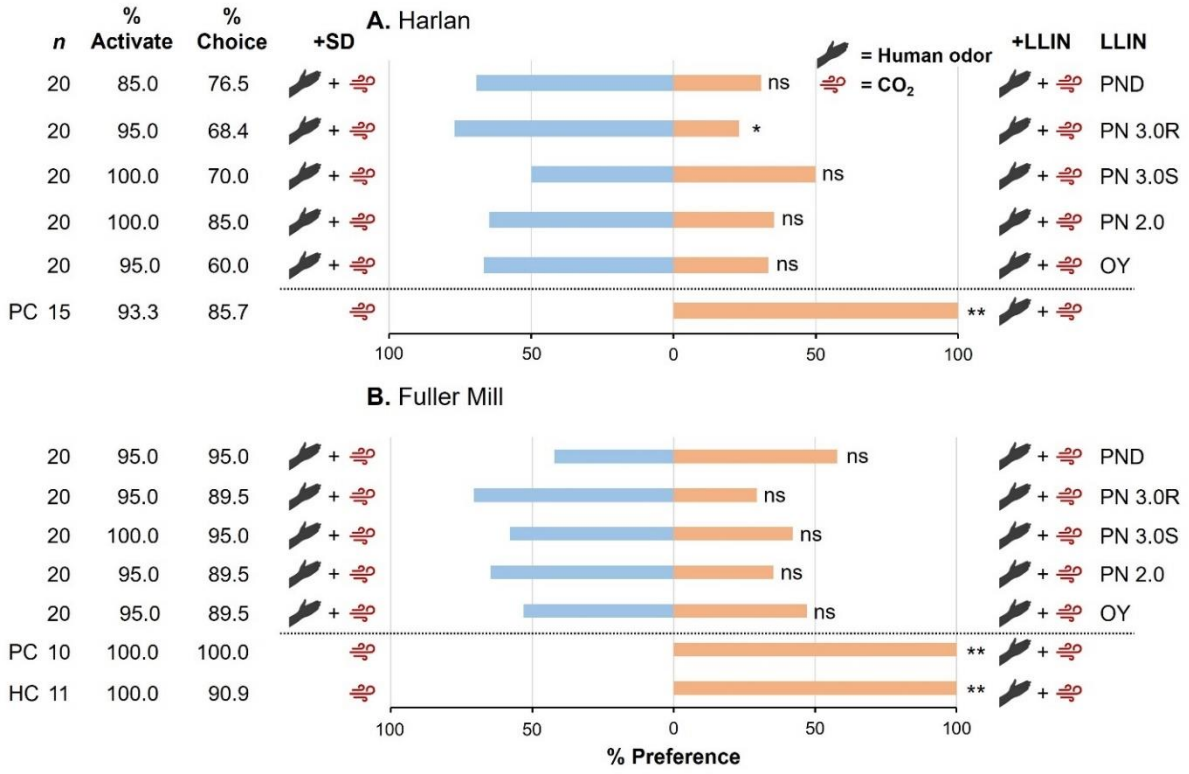

Fig. 4.3. Comparisons of olfaction plus contact-mediated behavioral responses of insecticide-susceptible (Harlan) (**A**) and insecticide-resistant (Fuller Mill) (**B**) *C. lectularius* to various LLINs. Individual adult females were assayed 10–14 d after ingesting a blood meal. The positive control (PC) and the Harlan control (HC in **B;** run daily alongside Fuller Mill PC) consisted of host cues (human skin odor and/or CO_2) at only one arm of the olfactometer. The percentages of activated bed bugs (% Activate) and activated bed bugs that chose either arm of the olfactometer (% Choice) are shown. The percentage preference toward subject stimuli, including LLIN (right side, orange), and towards the control stimuli, including the untreated Siam Dutch (SD) net (left side, blue) are shown. Percentage Activate was compared within each graph (one-way ANOVA) and the overall model for each was not significant ($P > 0.05$). Likewise, % Choice was compared within each graph (one-way ANOVA) and between strains (GLM), and both sets of comparisons did not reveal any significant differences ($P > 0.05$). Preference was compared independently for each LLIN by Chi-square test, with asterisks representing significant differences in preference denoted by *, $P < 0.05$; and **, $P < 0.01$.

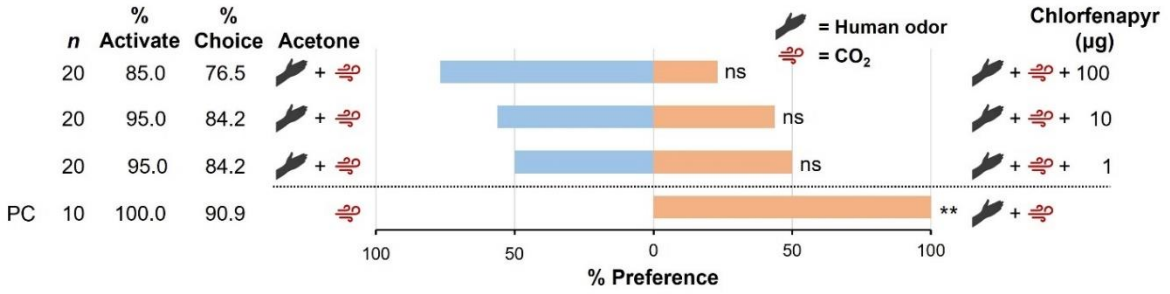

Fig. 4.4. Comparison of olfaction-mediated behavioral responses of insecticide-susceptible (Harlan) *C. lectularius* to scaling doses of chlorfenapyr. Individual adult females were assayed 10–14 d after a blood meal. The positive control (PC) consisted of host cues (human skin odor and/or CO_2) at only one arm of the olfactometer. The percentage of activated bed bugs (% Activate) and bed bugs that chose either arm of the olfactometer (% Choice) are shown. The percentage Preference toward subject stimuli, including chlorfenapyr dose (right side, orange), and towards the control stimuli, including acetone-treated paper (left side, blue) are shown. Percentage Activate and % Choice were compared separately (one-way ANOVA) and the overall model for each was not significant ($P > 0.05$). Preference was compared independently at each dose by Chi-square test, with asterisks representing significant differences in preference denoted by *, $P < 0.05$; and **, $P < 0.01$.

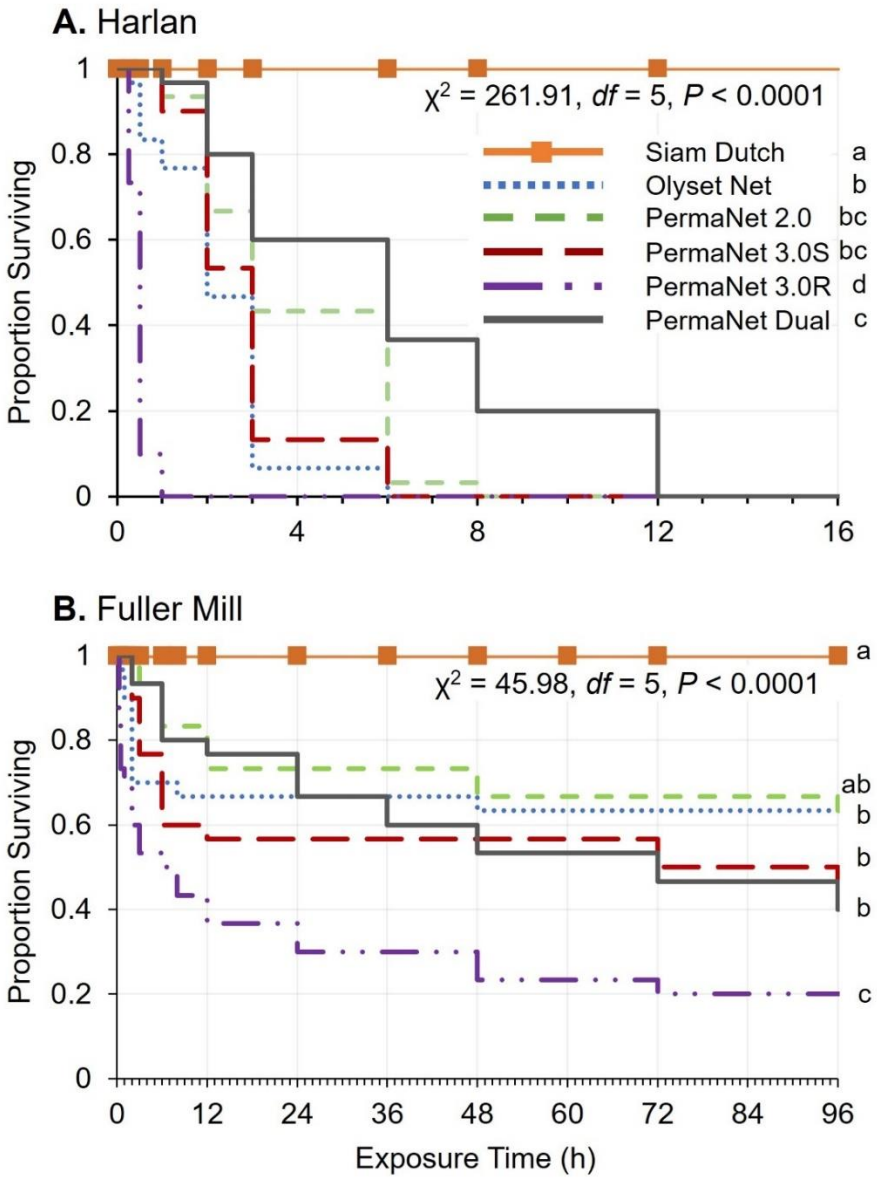

Fig. 4.5. Comparison of insecticide-susceptible (Harlan) (**A**) and insecticide-resistant (Fuller Mill) (**B**) *C. lectularius* surviving up to 96 h continuous contact with LLINs. For each of three replicates with each LLIN (n = 30 bed bugs per LLIN per strain), a group of 10 males, 4 days post-feeding, was placed on a 15.9 cm^2 circle of LLIN. Mortality, defined as bed bugs being unable to move with gentle prodding and then unable to flip back to their ventral side when inverted onto their dorsal side, was scored at 0.25, 0.5, 1, 2, 3, 6, 8, 12 h and then every 12 h to 96 h. Assays were conducted at 35–45% relative humidity, 25°C on a 12:12 (L:D) h cycle. Comparison of proportion surviving across all LLINs was done using Kaplan-Meier tests, and log-rank tests with Sidak correction for multiple comparisons. *P*-values are reported, and strain-specific LLIN comparisons sharing lower-case letters are not significantly different.