ORIGINAL PAPER

Decade long upsurge in mutations associated with pyrethroid resistance in bed bug populations in the USA

Cari D. Lewis[1] · Brenna A. Levine[1,2] · Coby Schal[3] · Edward L. Vargo[4] · Warren Booth[1]

Abstract

Over the past three decades, the bed bug *Cimex lectularius* has resurged as a prominent indoor pest on a global scale. Knockdown-associated insecticide resistance (*kdr*) involving the voltage-gated sodium channel, targeted by organochlorine and pyrethroid insecticides, was first reported in *C. lectularius* within a few years of the widespread use of dichlorodiphenyltrichloroethane (DDT) and has been implicated as a significant factor contributing to the species' recent resurgence. Since then, selection with pyrethroid insecticides has intensified, yet little is known regarding its short-term impacts on the frequency of *kdr*-associated mutations. Here, we report temporal changes in the frequencies of three *kdr*-associated mutations in *C. lectularius* populations collected across the USA from two time periods, sampled approximately a decade apart. The results reveal a significant increase in the frequencies of *kdr*-associated mutations over this period and the absence of the insecticide-susceptible genotype in recent collections. Furthermore, a significant transition was observed toward infestations possessing multiple *kdr*-associated mutations. These findings suggest that the persistent use of pyrethroid insecticides over the past decade continues to impose strong selection pressure on *C. lectularius* populations, driving the proliferation of *kdr*-associated mutations. They demonstrate that, if unabated, strong anthropogenic selection can drive the rapid evolution of adaptive traits.

Keywords *Cimex lectularius* · Knockdown resistance · Voltage-gated sodium channel · Urban evolution

Key message

- Lacking from the literature, however, are empiracal data documenting how these selection pressures alter the phenotypes or genotypes upon which they act over short-term temporal scales.

✉ Warren Booth
warren-booth@utulsa.edu

1 Department of Biological Science, The University of Tulsa, Tulsa, OK 74104, USA

2 Dorothy and George Hennings College of Science, Mathematics and Technology, Kean University, Union, NJ 07083, USA

3 Department of Entomology and Plant Pathology, North Carolina State University, Raleigh, NC 27695, USA

4 Department of Entomology, Texas A&M University, College Station, TX 77804, USA

Introduction

Globally, urban areas are undergoing rapid expansion and are increasing in density (Homer et al. 2020), promoting the establishment and spread of urban-adapted invasive species (Francis and Chadwick 2015; Reed et al. 2020). Within these anthropogenic environments, a myriad of selection pressures are exerted on resident populations, often resulting in the rapid evolution of phenotypes and genotypes (Johnson and Munshi-South 2017; Beans 2019). Ranging from the evolution of unique personality traits and morphologies, to genotypes that confer resistance to pesticides, many such adaptations have been shown to be beneficial, promoting population persistence and spread (Wada-Katsumata et al. 2013; Donihue and Lambert 2015; Winchell et al. 2016; Johnson and Munshi-South 2017; Zalucki and Furlong 2017).

Some pest species share a long history of anthropogenic association (Aplin et al. 2011; Puckett et al. 2016; Potter 2018; Tang et al. 2019). They are readily transported, inadvertently by humans, adapt well to the human-built

environment, and are of public health, veterinary, and economic significance (Brenner 1995; Corrigan 2011; Doggett et al. 2018; Hwang et al. 2018; Perron et al. 2018). As such, these pests have been the target of management efforts using various pesticide formulations (Rose 2001; Corrigan 2011; Potter 2011; Lee et al. 2018). The persistent use of pesticides exerts selection pressures that can drive the evolution of a suite of resistance-conferring adaptive mechanisms, including mutations in receptor target sites that reduce sensitivity to certain pesticides [e.g., knockdown resistance (*kdr*)] (Davies et al. 2008; Dong et al. 2014).

One group of insecticides, the sodium channel modulators, which includes the organochlorines (e.g., dichlorodiphenyl trichloroethane [DDT]), pyrethrins, and pyrethroids, alter the activity of *para*-type voltage-gated sodium channels (VGSCs), which are necessary for neuronal function. These insecticides maintain the sodium channels in an activated state, preventing cellular repolarization, resulting in paralysis and death (Soderlund and Knipple 2003). Nonsynonymous mutations within the VGSC gene have been identified that reduce *kdr*-associated target-site sensitivity to these insecticides across a variety of arthropod pests (Milani 1956; Martinez-Torres et al. 1998; Soderlund and Knipple 2003; Matowo et al. 2017). Despite being well-documented, temporal data on the distribution and frequency of *kdr*-associated mutations are surprisingly lacking for urban pest species in general, with the exception of *Anopheles* and *Aedes* mosquitoes (Mathias et al. 2011; Abdalla et al. 2014; Linss et al. 2014; Aguirre-Obando et al. 2016; Foster et al. 2016; Ismail et al. 2018; Amoudji et al. 2019; Biduda et al. 2019; Mandeng et al. 2019; Tancredi et al. 2020; Baltzegar et al. 2021) and house flies (Freeman et al. 2019). The lack of understanding of the evolutionary response of populations to the prolonged use of these insecticides may impede the continued development of effective control strategies over time.

The common bed bug, *C. lectularius*, is an obligate hematophagous ectoparasite, primarily of humans, that has re-emerged in recent decades to become a prominent indoor pest on a near global scale. This resurgence has been aided by an increase in travel (Potter et al. 2010), exchange of secondhand furniture, ineffective pest control, and the evolution of mechanisms conferring resistance to insecticides (Boase 2001; Doggett et al. 2004; Romero et al. 2007; Reinhardt et al. 2008; Zhu et al. 2010; Gordon et al. 2014). Within *C. lectularius* populations, various insecticide resistance mechanisms have evolved (Dang et al. 2017), with *kdr* mutations particularly well documented. First reported shortly after the widespread implementation of DDT (Busvine 1958), *kdr* mutations have been reported in populations around the globe (Romero et al. 2007; Yoon et al. 2008; Seong et al. 2010; Tawatsin et al. 2011; Wang and Wen 2011; Durand et al. 2012; Tomita et al. 2012; Booth et al. 2015; Dang et al. 2015; Lilly et al. 2015, 2017; Palenchar et al. 2015;

Raab et al. 2016; Balvin and Booth 2018; Holleman et al. 2019; Cho et al. 2020; Vander Pan et al. 2020; Akhoundi et al. 2021). Nevertheless, organochlorines, pyrethroids, and pyrethrins remain commonly used insecticides on a global scale. Within the USA, organochlorine-based pesticides were largely banned during the 1970s and 1980s. Pyrethroids, in contrast, remain among the most widely used insecticides. Active ingredients that have been evaluated include alpha-cypermethrin, bifenthrin, cypermethrin, cyfluthrin, cyphenothrin, pyrethrin, esfenvalerate, etofenprox, imiprothrin, lambdacyhalothrin, d-phenothrin, phenothrin, and d-tetramethrin; however, deltamethrin and permethrin are most frequently employed (Lee et al. 2018). Given their widespread use in formulations that range from insecticide-impregnated mattress liners to liquid sprays, this insecticide class persists as a selection pressure potentially promoting *kdr*-associated mutations that confer resistance to these insecticides.

In *C. lectularius*, three nonsynonymous mutations in the VGSC-gene (V419L, L925I, and I936F) have been found to be associated with *kdr* resistance (Yoon et al. 2008; Dang et al. 2015). The population frequency and geographic distribution of these mutations have been reported from a variety of locations, including the USA (Yoon et al. 2008; Zhu et al. 2010; Raab et al. 2016; Holleman et al. 2019), Europe (Durand et al. 2012; Booth et al. 2015; Balvin and Booth 2018; Vander Pan et al. 2020; Akhoundi et al. 2021), the Middle East (Palenchar et al. 2015), Asia (Seong et al. 2010; Tomita et al. 2012; Cho et al. 2020), and Australia (Dang et al. 2015). However, we are unaware of any studies that assessed temporal changes in the frequencies of *kdr*-associated mutations at any landscape level. Although it would be most instructive to track the frequency of *kdr* mutations over time within the same populations, this design is particularly challenging with residential infestations due to their ephemerality and turnover of residents. Here, we report temporal changes in the frequency of the three *kdr*-associated mutations from populations of *C. lectularius* collected across the USA at two time periods approximately 10 years apart. We hypothesized that both the number of infestations exhibiting *kdr*-associated mutations, and the number of adaptive mutations per individual bed bug would increase over time, due to selection imposed by the frequent and widespread use of pyrethrin and pyrethroid insecticides.

Materials and methods

Bed bugs were collected during two separate time periods: August 30, 2005, to December 20, 2009 (electronic supplementary material, Table S1), and June 27, 2018, to December 27, 2019 (electronic supplementary material, Table S2), representing 394 unique populations (161 in 2005−2009

Table 1 Distribution of *Cimex lectularius* populations by state for each sampling period

| State	2005–2009	2018–2019
Alabama	1	-
Alaska	–	3
Arizona	1	–
Arkansas	2	–
California	7	27
Colorado	3	–
Florida	13	10
Georgia	2	2
Hawaii	1	–
Idaho	–	1
Illinois	3	79
Indiana	5	4
Iowa	5	–
Kansas	1	5
Kentucky	3	–
Louisiana	1	–
Maine	1	–
Maryland	4	–
Massachusetts	2	–
Michigan	3	–
Minnesota	1	–
Missouri	1	4
New Jersey	12	20
New York	3	1
Nebraska	3	–
Nevada	5	–
North Carolina	16	–
Ohio	20	33
Oklahoma	3	9
Oregon	4	15
Pennsylvania	17	5
South Carolina	1	–
Tennessee	–	1
Texas	4	13
Utah	3	–
Virginia	5	–
Washington	3	–
Wisconsin	2	1
Total	161	233

and 233 in 2018−2019). Collections spanned 38 US states (Table 1, Fig. 1a) and were made by pest management companies (electronic supplementary material, Table S3). Upon collection, specimens were preserved in 100% ethanol and stored at −20 °C until DNA extraction. Genomic DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (QIAGEN, Germantown, MD).

Due to the highly inbred nature of *C. lectularius* populations (Booth et al. 2012, 2018; Saenz et al. 2012), which results from population foundation by a single gravid female or small group of highly related individuals, the genetic diversity and hence *kdr*-associated mutation profile of a given population can be determined through the sequencing of a single individual. Therefore, each population is represented here by a single specimen. Two genomic fragments that contain the three *kdr*-associated mutations were PCR-amplified and sequenced using primer combinations BBParaF1/BBParaR1 (V419L) and BBParaF3/BBParaR3 (L925I, I936F), following the methods of Holleman et al. (2019). The resulting chromatograms were visualized and scored using CLC Genomics Workbench (QIAGEN). Knockdown resistance genotype designations are reported as follows: wild type (pyrethroid-susceptible in the absence of other resistance mechanisms; L925, V419, I936), L925I (mutant L925I [leucine (L) replaced by isoleucine (I)], wild type V419&I936), L925I&V419L (mutations in regions L925I and V419L [valine (V) replaced by leucine (L)], wild type I936), L925I&I936F (mutations in both L925I and I936F [isoleucine (I) replaced by phenylalanine (F)], wild type V419), and I936F (mutant I936F, wild type L925&V419). The V419L (wild type L925, I936, mutant V419L) genotype was not detected in any population screened. Where the mutations are heterozygous, these are reported with a superscript "*het*".

Genotype frequencies were compared using Chi-square tests. Additionally, the overall homozygous genotype frequencies of populations present in the same US states across both sample periods were compared.

Results

All samples yielded unambiguous sequences for both PCR fragments. Of the 161 populations sampled between 2005 and 2009, genotypes L925I and L925I&V419L accounted for 36% and 50% of the populations, respectively (Table 2 and Fig. 1a). The wild type ('susceptible') genotype was found in 2.5% of the populations. Approximately 10% of samples screened were found to be L925I&V419L[het], while the I936F, L925I[het]&I936F[het], and I936F[het] genotypes each comprised < 0.1% of the remaining infestations (Table 2 and Fig. 1a). In contrast, genotype L925I&V419L predominated in samples collected between 2018 and 2019, accounting for 84% of the populations, while genotypes L925I (~ 5%), L925I[het] (~ 1%), L925I&V419L[het] (~ 9%), and L925I[het]&V419L (~ 1%) comprised the remaining populations (Table 2 and Fig. 1a). The wild type and I936F genotypes were not detected in the 2018−2019 populations.
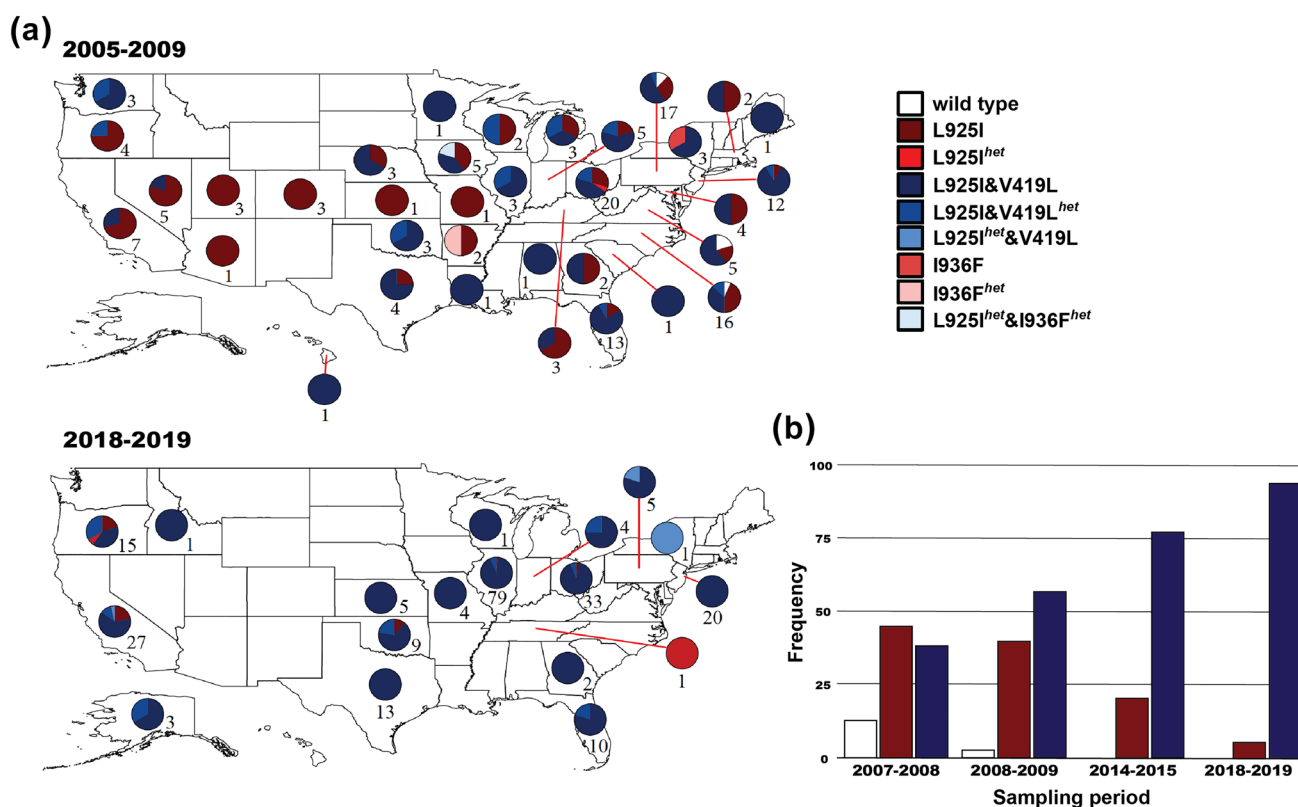
Fig. 1 a Genotype distribution of *kdr*-associated mutations from *Cimex lectularius* sampled across the U.S. Pies represent genotype frequencies within each state and the number below each indicates the number of populations sampled. **b** Change in genotype frequencies over time of three homozygous genotypes—wild type, 925, 925&419. Colors correspond to genotypes in **a**. 2007–2008 data from Zhu et al. (2010), 2014–2015 from Holleman et al. (2019)

Temporally, there were significant differences in the frequencies of all homozygous [wild type, $p < 0.05$; L925I, $p < 0.001$; L925I&V419L, $p < 0.001$), but not the heterozygous genotypes and I936F, likely due to their low frequency across datasets (Table 2). Comparisons conducted using only populations sampled in the same US states in both timeframes again revealed significant differences in the frequencies of all homozygous genotypes (electronic supplementary material, Table S4).

Discussion

By surveying 394 distinct populations of *C. lectularius* across the USA, collected from two time periods separated by approximately a decade, this study represents the most comprehensive screening to date of *kdr*-associated mutation frequencies for the species. To our knowledge, this is also the first temporal comparison of *kdr* mutation frequencies in any urban pest insect in the U.S., outside of house flies collected from animal production facilities (Freeman et al. 2019) and mosquitoes, which were sampled in Asia, Africa, and South America, but not in the U.S. (Mathias et al. 2011; Abdalla

et al. 2014; Linss et al. 2014; Aguirre-Obando et al. 2016; Foster et al. 2016; Ismail et al. 2018; Amoudji et al. 2019; Biduda et al. 2019; Mandeng et al. 2019; Tancredi et al. 2020; Baltzegar et al. 2021). Comparing our homozygous populations from 2005 to 2009 ($n = 143$) to those of Zhu et al. (2010) [$n = 93$] revealed significant differences in *kdr* genotype frequencies (wild type: $\chi^2(1, n = 236) = 9.11$, $p < 0.05$; L925I: $\chi^2(1, n = 236) = 0.45$, $p > 0.05$; L925I&V419L: $\chi^2(1, n = 236) = 7.25$, $p < 0.05$; V419L: $\chi^2(1, n = 236) = 4.67$, $p < 0.05$), supporting the lower baseline frequency of *kdr* mutations present in the USA in 2006–2007 (Zhu et al. 2010), and a reduction in wild type and increase in L925I&V419L by 2008–2009. Prior to our study, the most recent study conducted in the U.S. reported *kdr* genotype frequencies for populations collected across Midwestern states between 2014 and 2015 (Holleman et al. 2019), with the frequencies of L925I (~21%) and L925I&V419L (~76%) intermediate between the datasets presented here (Fig. 1b), as expected for a shift toward increased resistance. Thus, our results, when viewed together with those of Zhu et al. (2010) and Holleman et al (2019), reveal a significant increase in the frequency of *kdr* mutations over approximately a decade, with a shift toward multiple *kdr*

Table 2 Frequency of *kdr*-associated genotypes reported in *Cimex lectularius* populations sampled in the U.S. sampled between 2005–2009 and 2018–2019

Sample periods	No. of States	No. of pops	No. of individuals	*kdr* genotypes								
				Wild type	L925I	L925I^{het}	L925I&V419L	L925I^{het}&V419L	L925I&V419L^{het}	1936F	1936F^{het}	L925I^{het} & 1936F^{het}
2005–2009	35	161	161	4 (0.02)	57 (0.35)	1 (0.006)	81 (0.50)	16 (0.10)	0 (0.00)	1 (0.006)	1 (0.006)	1 (0.006)
2018–2019	18	233	233	0 (0.00) [5.85]*	12 (0.05) [60.32]**	2 (0.01) [0.07]	194 (0.84) [49.04]**	22 (0.09) [0.03]	3 (0.01) [2.09]	0 (0.00) [1.45]	0 (0.00) [1.45]	0 (0.00) [1.45]

For each genotype, the number of observations is reported. Parentheses indicate the frequency of each genotype and the brackets report the χ^2 statistic where $\chi^2(1, n = 394) = |\chi^2$ stat$|$, $p > 0.05$ or *$p < 0.05$, **$p < 0.001$

mutations per population (Fig. 1b). Given the sample sizes represented here, with the 2018–2019 dataset comprising 233 infestations [~ 31% more than the 2005–2009 dataset ($n = 161$), which is > twofold the populations reported in Zhu et al. (2010) ($n = 93$)], the failure to detect the wild-type genotype among the infestations sampled is not likely to be an artifact of sample size, but instead the actual reduction in the frequency of the genotype among infestations. This is likely in response to persistent selection driven by the long-term use of pyrethroid insecticides.

Prior bioassay-based research in *C. lectularius* has linked genotypes L925I and L925I&V419L to increased levels of pyrethroid resistance. Evaluating 17 populations collected across the U.S. between 2006 and 2009, only three were found to be susceptible following treatment with a discriminating dose of technical grade deltamethrin, resulting in 100% mortality (Zhu et al. 2010). Of the 14 that exhibited resistance, 13 were found to possess either the L925I mutation or both L925I and V419L mutations (Zhu et al. 2010). Prior to this, Romero et al. (2007) assessed the resistance status of four field-derived colonies initially collected from Lexington, KY, and Cincinnati, OH, alongside two lab colonies, not previously exposed to pyrethroid insecticides in the 20–30 years since colony establishment, through laboratory bioassays. Following exposure to two pyrethroid insecticides, deltamethrin and λ-cyhalothrin, field-derived colonies from KY and OH were found to exhibit extremely high levels of resistance, including > 12,765-fold deltamethrin resistance, compared to a susceptible wildtype population (Romero et al. 2007). Crosses between field-derived resistant populations and laboratory-derived susceptible strains exhibited intermediate levels of resistance. The *kdr* mutation profile of these populations was not determined. Globally, reports of pyrethroid-resistant *C. lectularius* populations are common (see Dang et al. 2017). Some of these studies include assessments of *kdr* mutation profiles; however, temporal data are missing and studies that link *kdr* mutation frequency with pyrethroid resistance are limited. That said, the absence of *kdr* mutations in bat-associated *C. lectularius* populations sampled in Europe, which have not been exposed to pyrethroid insecticides (Booth et al. 2015; Balvin and Booth 2018), suggests that pyrethroid insecticides impose strong selection for *kdr* mutations in urban bed bug populations. In addition, low frequencies of *kdr* mutations in bed bug populations sampled in Australia between 1994 and 2002, and their increase in frequency around the globe since the early 2000s (See Holleman et al. 2019), strongly suggest that pyrethroid resistance has increased in recent decades.

Comparable findings of temporal changes in *kdr* mutations have been reported in mosquito populations exposed to the prolonged use of insecticides. Congruent with the results presented here, several long-term studies (> 10 years) showed an increase in *kdr* mutation frequencies

in populations of *Anopheles gambiae* (Mathias et al. 2011) and *Aedes aegypti* (Linns et al. 2014; Baltzegar et al. 2021), from different geographic regions. Recently, Baltzegar et al (2021), documented the complete replacement of the wild type pyrethroid-susceptible genotype, with either single or double *kdr*-mutants in *Ae. aegypti* mosquitos sampled in Iquitos, Peru, with the replacement occurring over a period of 13 years following the introduction of a city-wide pyrethroid insecticide control strategy. Similarly, the malaria vector, *An. arabiensis*, was shown to respond to strong selection for deltamethrin resistance over 4 years, which was correlated with the L1014F *kdr* mutation (Ismail et al. 2018). Verhaeghen et al. (2010) documented a 5-year increase in *kdr* mutations in *An. gambiae* from multiple geographic locations, and similar region-specific fluctuations in *kdr* mutation frequencies have been shown in *Ae. albopictus* at multiple time intervals (Tancredi et al. 2020). A significant increase was also reported for the 1b resistance allele in house flies collected over a 10-year period from animal production facilities across five U.S. states (Freeman et al. 2019). While these studies span different regional pest management practices, the trend of increasing *kdr* mutation frequencies is consistent. However, a unique characteristic of *C. lectularius* is the high level of intra-population inbreeding, and an apparent lack of significant levels of gene flow (Booth et al. 2012, 2015, 2018; Saenz et al. 2012; Fountain et al. 2014). As a result, resistance alleles rapidly propagate and are then spread in a fixed-state through propagule transfer. While there is still uncertainty surrounding the fitness costs of resistance in *C. lectularius* (Polanco et al. 2011; Gordon et al. 2015), the frequency and distribution of *kdr* mutations and the prevalence of the species suggests this may be minimal.

The zygosity of *kdr* mutations and its impact on insecticide resistance adds additional complexity. For example, in the house fly, *Musca domestica,* mutations exhibit a recessive pattern of inheritance (Rinkevich et al. 2012). In contrast, polygenic modes of inheritance have been documented in the codling moth, *Cydia pomonella* (Bouvier et al. 2001), with both recessive and incomplete dominance described. Unlike Zhu et al. (2010), our study reports the zygosity of the *kdr*-associated mutations detected. This information may be relevant for control, for while the level of resistance exhibited by *C. lectularius* heterozygous for a given mutation is unclear, it has been suggested that heterozygotes may show incomplete dominance (Romero et al. 2007). Heterozygotes may therefore exhibit intermediate levels of resistance between wild type and homozygous mutants, although this has yet to be confirmed. If *kdr* exhibits incomplete dominance in *C. lectularius*, heterozygotes would be expected in areas undergoing genotype replacement from wild type to resistant, either through population spread or continued pyrethroid applications. However, bed bugs heterozygous for *kdr* mutations would also be expected to succumb to control more rapidly than homozygous mutant individuals. In both time periods sampled, L925I&V419F^{het} was the most common heterozygous genotype observed, representing 89% (2005–2009) and 78% (2018–2019) of the heterozygous individuals. Given the short generation time of *C. lectularius*, high fecundity, and lack of obvious inbreeding depression (Fountain et al. 2014), the transition within a population to the double homozygous mutant (L925I&V419L) would be expected to occur rapidly following selection by insecticide treatment.

In summary, the significant temporal shift toward increased frequencies of *kdr* mutations reveals how anthropogenic selection can influence the evolution of a species of public health, veterinary, and economic significance, and suggests that indoor pest insects may represent ideal model systems to study the effects of anthropogenic influences on the evolution of adaptive traits in urban species. The presence of multiple *kdr* mutations has been associated with elevated levels of pyrethroid resistance in other pest insects (Soderlund 2008; Abdalla et al. 2014). Thus, we presume that the higher proportion of the bed bugs collected in 2018–2019 that possess *kdr* mutations reflects a greater resistance to pyrethroids than those collected a decade earlier, resulting from selection for pyrethroid resistance. Therefore, our findings are practically significant in the development of management strategies for the control of *C. lectularius* populations and those of other urban and indoor pest species commonly controlled with insecticides. Given the general trend of increasing reports of pyrethroid resistance in urban and indoor pests, pyrethroid insecticides alone are having limited impact on the control of these populations. Furthermore, while bed bug populations theoretically may lose *kdr* mutations in the absence of pyrethroid exposure, with reversion back to pre-selection profiles possibly resulting from fitness costs associated with resistance (Gordon et al. 2015), the ability to do so relies on the presence of susceptible alleles within the population. Our results show that these susceptible alleles are rapidly being lost from populations across the U.S.; thus, reversion to a susceptible genotype will require mutation and subsequent spread in the absence of control. As such, these data support the need for insecticides to be composed of blends of insecticide classes (e.g., pyrethroids and neonicotinoids) (Lee et al. 2018), in addition to integrated approaches that enable early detection and non-chemical control methods (e.g., heat treatment) (Kells 2018).

Authors' contributions

CDL, WB, BAL, ELV, and CS designed the experiment. CDL and WB collected and analyzed the data. All authors contributed to the writing of the manuscript and gave final approval for publication.

Availability of Data and Material Data supporting the results can be found in the Online Supplementary Material.

Code Availability Not applicable.

Declarations

Conflict of interest The authors declare no conflicts of interest.

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