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Unique features of a global human ectoparasite identified through sequencing of the bed bug genome

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The bed bug, *Cimex lectularius*, has re-established itself as a ubiquitous human ectoparasite throughout much of the world during the past two decades. This global resurgence is likely linked to increased international travel and commerce in addition to widespread insecticide resistance. Analyses of the *C. lectularius* sequenced genome (650 Mb) and 14,220 predicted protein-coding genes provide a comprehensive representation of genes that are linked to traumatic insemination, a reduced chemosensory repertoire of genes related to obligate hematophagy, host–symbiont interactions, and several mechanisms of insecticide resistance. In addition, we document the presence of multiple putative lateral gene transfer events. Genome sequencing and annotation establish a solid foundation for future research on mechanisms of insecticide resistance, human–bed bug and symbiont–bed bug associations, and unique features of bed bug biology that contribute to the unprecedented success of *C. lectularius* as a human ectoparasite.

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The common bed bug, *Cimex lectularius*, has a 3,000-year documented association with humans that is likely much more ancient[1]. This species was nearly eradicated after World War II in most economically and politically stable countries, in part through the liberal use of pesticides[2], but reservoir populations have remained in underdeveloped countries, disadvantaged communities and in association with bats, chickens and other animals[2]. During the past ~20 years, however, there has been a global resurgence of bed bugs in every continent except Antarctica[2]. The upsurge in prevalence of bed bugs has been extraordinary with infestations increasing 4,500% in Australia and similar escalations in other regions[2,3]. Bed bugs have also become highly prolific in the United States, with reports of infestations in all 50 states[2,3]. This rapid expansion has been linked to increased international travel, frequent exchange of second-hand materials, a lack of education on issues related to bed bugs and the evolution of resistance to all major classes of insecticides, including organochlorines, organophosphates and pyrethroids[4].

The biology of bed bugs features many unique aspects that contribute to their success as a human parasite[5]. First, all mobile life stages of the bed bug are obligatory blood feeders, and blood serves as the sole source for ingested nutrients and water[5,6]. This trophic specialization requires a dedicated chemosensory system to detect, find and accept proper hosts. Moreover, recent evidence of two distinct lineages of *C. lectularius*, one associated with humans and the other with bats[7], suggests chemosensory specialization between bat- and human-associated bed bugs. In addition, hematophagy requires specific enzymes and associated pathways to properly digest and assimilate blood and dispose of excess water; such specialization also drives obligate associations with symbionts, including *Wolbachia*, that generate critical micronutrients that are deficient in vertebrate blood[8,9].

Bed bugs mate through traumatic insemination; males pierce the cuticle of the female abdomen with a modified reproductive organ and deliver sperm into her haemolymph[10,11]. This mode of reproduction is under strong selection by sexual conflict that involves sexually transmitted microbes and selects for immune networks that may in turn affect bed bug–pathogen associations[12]. Bed bugs appear resistant to the effects of repeated rounds of inbreeding[13], which likely results in the fixation of beneficial gene complexes and the purging of deleterious alleles that may improve local adaptation, such as pesticide resistance. Interestingly, outbreeding does not appear to be disadvantageous, but any heterotic effect appears to be minimal and short-lived[13]. Bed bugs have recently been identified as potential vectors for American trypanosomes[14], but unlike other haematophagous arthropods, direct confirmation of pathogen transmission to humans is rare[15,16]. Last, resistance to insecticides has become widespread and pyrethroid resistance has reached levels 10,000-fold higher than in susceptible bed bug populations[17]. Multiple mechanisms of insecticide resistance and cross-resistance may also impede the development of new classes of pesticides. Recent transcriptome studies have examined other specific aspects of bed bug biology, but the lack of a sequenced genome has stalled deeper understanding of bed bugs and their evolutionary and ecological relationships relative to other insects.

Here we report the genome of *C. lectularius* and associated bacteria along with phylogenomic analyses and extensive manual annotation. This study reveals evolutionary adaptations associated with the lifestyle of bed bugs, including significant reductions in chemosensory genes, expansion of genes that are associated with blood digestion and the entire repertoire of genes that have been associated with pesticide resistance in various other species. In addition, we identified the presence of multiple putative lateral gene transfer (LGT) events from various bacteria, including *Wolbachia* and *Arsenophonus*.

Results and Discussion

General features of the genome and orthologue analyses. Individual features of the bed bug genome analyses are provided as Supplementary Information (Supplementary Figs 1–42; Supplementary Notes 1–22; Supplementary Data 1–33). Our final draft assembly comprises 650.47 Mb of total sequence in 1,402 scaffolds and 45,073 contigs (N50 lengths 7.17 Mb and 23.5 kb, respectively; Supplementary Data 1). This is 25% smaller than the predicted genome size of 864.5 Mb (determined through comparison with other insects by propidium iodide analyses; Supplementary Note 17) and is likely due to unassembled heterochromatin and other repetitive regions. We predicted 13,953 genes using a custom MAKER annotation pipeline tuned for arthropod genomes and this was improved to 14,220 through manual curation. A total of 1,352 gene models representing gene families of interest, including 273 cuticle proteins and 114 chemoreceptors were manually curated, confirming gene identity and revealing where automated gene structures needed correction (Supplementary Note 1). To assess the completeness of the assembly and gene prediction, we analysed the predicted genes and genome assembly for benchmarking sets of universal single-copy orthologues (BUSCOs[18]). In addition, the presence of a complete Hox cluster and all expected autophagy genes was documented, two categories that are known to be conserved among insect genomes (Supplementary Notes 10 and 12). In general, the *C. lectularius* gene set and genome has slightly more missing BUSCOs, ~10%, compared with the genomes of seven other arthropods, but is still relatively complete (Supplementary Data 28). We therefore concluded that the data set for *C. lectularius* is sufficiently comprehensive for further downstream analyses.

In addition, we characterized homologous and orthologous relationships between genes in relation to those of other sequenced arthropods using a previously described orthology delineation approach employed by OrthoDB[19]. The analyses were performed with the 45 arthropod species included in OrthoDB7 (http://www.orthodb.org). Over 80% of *C. lectularius* genes have orthologues in at least one arthropod species (Fig. 1). Of these, 1,734 were universal single-copy orthologues across eight species, which were used to determine the maximum-likelihood phylogeny. As expected, our analyses of these eight arthropod genomes placed another hemipteran, the pea aphid *Acyrthosiphon pisum*, as the sister species of *C. lectularius* (Fig. 1). It is worth noting that *A. pisum* has more than twice as many genes due to extensive gene duplication in >2,000 gene families[20]. Large-scale transcription factor analyses revealed 634 putative transcription factors and we were able to infer DNA-binding motifs for 214 (34%; Supplementary Note 22).

Host location, obligate blood feeding and immunity. Bed bugs are obligate blood feeders, and unlike mosquitoes and many other blood-feeding insects, all immature stages and both sexes of adults rely exclusively on blood for nutrition and water[5–7]. *C. lectularius* prefers humans as hosts but accepts a range of other vertebrate hosts[5,7]. The association with humans in the built environment, coupled with their crepuscular/nocturnal activity and the complete reduction in wings, predicts specialized mechanisms for host location, acceptance, and blood ingestion and digestion. Bed bugs are equipped with small compound eyes that protrude prominently from the lateral head capsule and object recognition is suspected to play a role in host detection[21]. Consistent with low-resolution landscape vision, the bed bug genome contains one member each of the ultraviolet- and broadband long-wavelength-sensitive rhabdomeric opsin subfamilies in line with that of most other hemipteran genomes

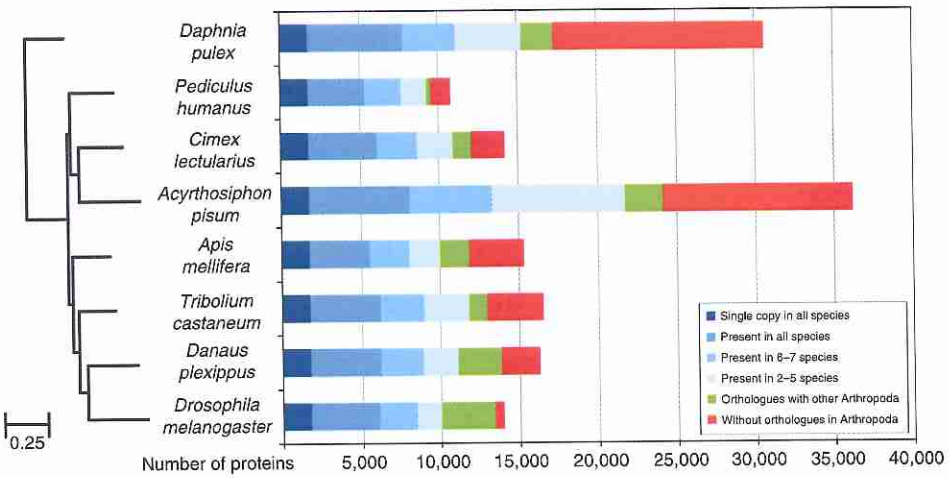

Figure 1 | Phylogenetic placement and orthology comparison among *Cimex lectularius* and other arthropod species. The phylogenetic analysis places *C. lectularius* as a sister species to another hemipteran, *Acyrthosiphon pisum*. The phylogeny is built using RAxML and it is based on the 1,734 single-copy orthologues that are present in all eight species. All nodes in the phylogenetic tree have 100% bootstrap support, while the branch length unit is substitutions per site. There are 1,734 genes that are present as single copy in all eight species tested. Another 4,187 of the *C. lectularius* genes are found in varying copy number in the other seven species, while 2,433 are found in the majority of species (that is, in 5–7 species) and 2,153 genes are found in ≥2 species (that is, in 2–4 species). Moreover, 1,147 genes have an orthologue in an arthropod other than the selected seven species. Last, 2,285 genes are lineage specific and do not have an orthologue in any other arthropod species.

sequenced (Supplementary Note 9), as well as crepuscular insect species in general[22]. Circadian clock genes in *C. lectularius* appear to encode both *Drosophila*- and mammalian-like proteins (Supplementary Note 5), with notable absence of sequences for CRY1 and JET, which are necessary in *Drosophila* for the light input pathway to the clock[23,24]. *Cimex* may thus represent a valuable model in circadian rhythm research, particularly for organisms that inhabit low-light or -dark environments.

Olfactory and gustatory processing in insect sensilla depends on three families of chemoreceptors: odourant, gustatory and ionotropic receptors[25]. Olfactory receptors play critical roles in mate finding, host location and navigation through a dark environment using the sense of smell. The major functions of gustatory receptors (GRs) are to mediate gustation—most importantly to detect sweet (phagostimulatory) and bitter (deterrent) tastants—as well as to sense carbon dioxide[26]. Ionotropic receptors evolved from ionotropic glutamate receptors in ancestral animals, and are involved in both olfaction and gustation[27]. We idenified 48 genes encoding 49 olfactory receptors, 24 genes encoding 36 GRs and 30 ionotropic receptor genes (Fig. 2a; Supplementary Note 4). This repertoire of chemosensory genes is substantially reduced relative to that of phytophagous hemipterans (for example, pea aphid), extending a similar trend noted in the genome sequences of other blood-feeding insects (Fig. 2a). Moreover, the intermediately sized repertoire of bed bug chemoreceptors is in line with the moderate complexity of its chemical ecology, being an obligate blood feeder such as tsetse flies (*Glossina morsitans*)[28,29], but having a broader host range that encompasses many vertebrates, whereas *Pediculus humanus humanus* (body louse) feeds only on humans[30]. We found no sugar receptors in the *Cimex* genome, as previously documented in other obligate blood feeders, including tsetse flies[28,29] and lice[30]. This finding also explains the lack of phagostimulation by glucose in *C. lectularius*[31]. Remarkably, *Cimex* has four GRs related to a conserved lineage of carbon dioxide receptors found in flies, moths, beetles and a termite[32], but that are absent from the pea aphid, hymenopteran species and blood feeders such as *Pediculus*[30] (Supplementary Note 4). The *Cimex* chemosensory gene families appear to have few expansions and slow evolving members (Supplementary Figs 2–4), suggesting a comparatively stable chemosensory ecology. We also found 11 odourant-binding proteins that appear to be highly species specific in *C. lectularius*, and 14 chemosensory proteins that are more conserved relative to other blood-feeding insects[33].

One of the major obstacles in the acquisition of a blood meal is host haemostasis, the physiological process that prevents blood loss through platelet aggregation, fibrin crosslinking, vasoconstriction and local immune responses. The bed bug genome builds upon previous sialotranscriptome and proteome studies[34] and contributes to our understanding of bed bug saliva complexity and unique adaptations of blood-sucking insects. Of interest, *Cimex* appears to have expanded salivary apyrases, proteins involved in the inhibition of ADP-dependent platelet aggregation, including two *Cimex*-type apyrases[35]. In addition, 12 members of the inositol polyphosphate phosphatase family that act as nitric oxide carriers, and 6 members of the Ap4a_hydrolase family, the largest number in any insect genome, were identified (Fig. 2b; Supplementary Note 18). This expanded array of salivary proteins likely permits bed bugs to stealthily feed repeatedly on the same host without inflicting pain.

Vertebrate blood is an excellent source of proteins and lipids, but it is deficient in specific micronutrients, has high water content, and its digestion requires a suite of specific digestive enzymes (Fig. 2c). Analysis of the *C. lectularius* genome revealed 187 potential digestive enzymes (Supplementary Data 12). *C. lectularius* has fewer serine proteases than most insects, but a similar repertoire to blood-feeding *Rhodnius* (kissing bug) and *Pediculus* (Supplementary Note 7 (refs 30,36)). Of interest is a large expansion of genes associated with cathepsin D (Fig. 2c), an aspartic protease adapted for acidic pH[35]. A similar expansion, albeit of different specific cathepsin D genes, has been found in *Rhodnius* and deemed critical for optimal blood digestion[36,37].

Removal of excess water from the blood meal is essential for proper digestion and aquaporins (AQPs) appear to be critical for this process[38]. Bed bugs possess seven or eight AQP genes, which are within the 6–8 range common for most insects[38] (Supplementary Note 3). Among peptide hormones and amine receptors, we documented a full complement of diuretic and antidiuretic hormones and their receptors that serve to precisely initiate and terminate postprandial diuresis (Supplementary Note

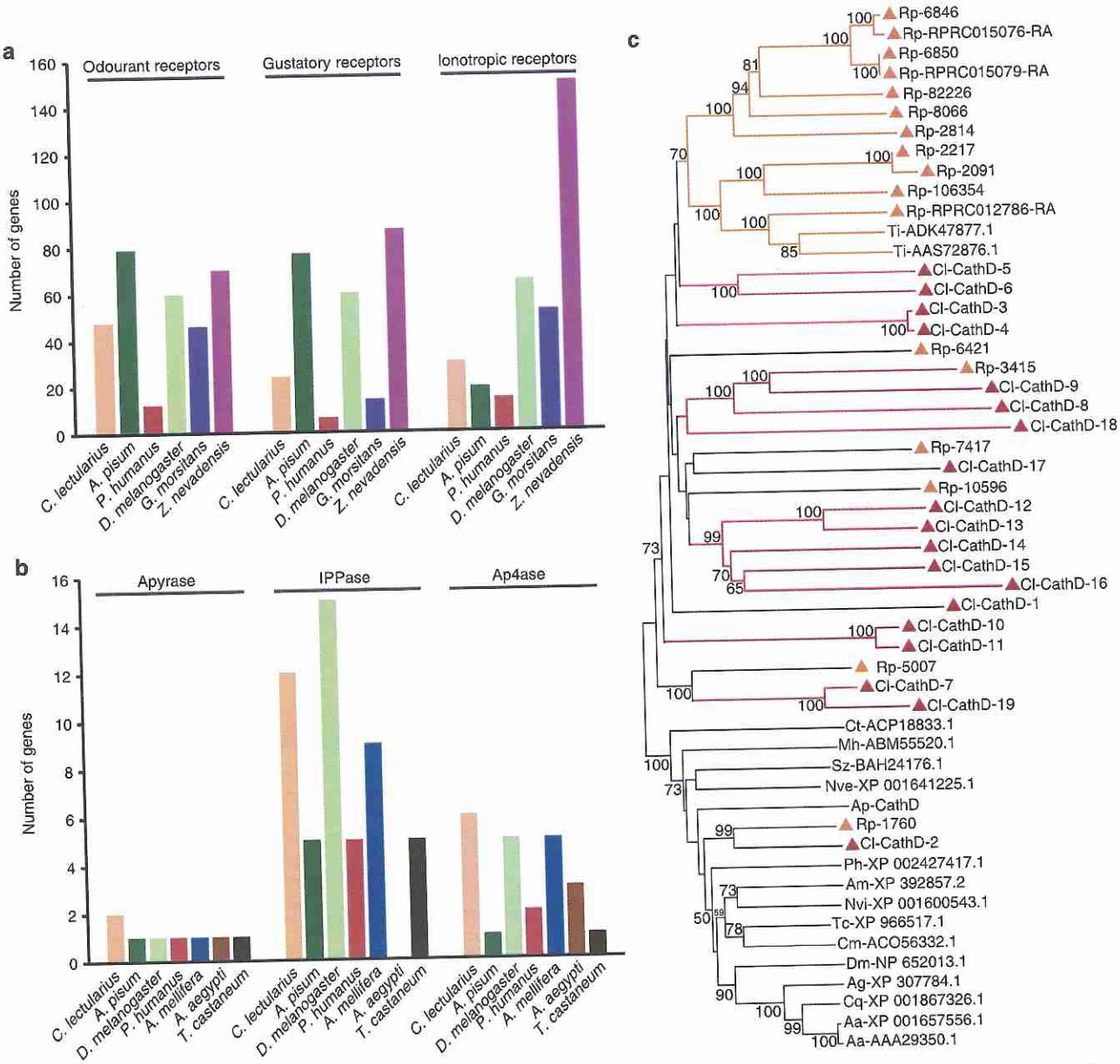

Figure 2 | Aspects related to host location and blood feeding identified based on the *C. lectularius* genome. (**a**) Genes associated with chemical reception among multiple insect species. *Zootermopsis nevadensis* (**b**) Genes associated with saliva function among multiple insect species. (**c**) Phylogeny of cathepsin D genes among multiple insect species. Sequences derived from *Cimex lectularius* (Cl) are denoted with red triangles and those derived from *Rhodnius prolixus* (Rp) are denoted with orange triangles. Other insect cathepsin D proteins represent those of *Triatoma infestans* (Ti), *Acyrthosiphon pisum* (Ap), *Anopheles gambiae* (Ag), *Drosophila melanogaster* (Dm), *Pediculus humanus corporis* (Ph), *Apis mellifera* (Am), *Nasonia vitripennis* (Nvi), *Tribolium castaneum* (Tc), *Callosobruchus maculatus* (Cm), *Sitophilus zeamais* (Sz), *Chrysomela tremula* (Ct), *Maconellicoccus hirsutus* (Mh), *Nematostella vectensis* (Nve), *Culex quinquefasciatus* (Cq) and *Aedes aegypti* (Aa).

14). Unlike *Rhodnius*[39,40], *C. lectularius* has only one *capa* gene encoding antidiuretic neuropeptide hormone.

Like blood-feeding ticks and triatomine bugs, but unlike most other blood-feeding insects, bed bugs can survive long periods of starvation between blood meals[5,6]. This adaptation requires nutrient conservation (for example, lower metabolism) and mechanisms to prevent excessive water loss and dehydration-induced mortality[5,6]. The latter is specifically dependent on differential expression of aquaporins and heat-shock proteins[38] (Supplementary Notes 3 and 11). In general, genes for heat-shock proteins and autophagy are similar in *Cimex* and other insect species, suggesting that their differential expression is likely responsible for the extreme dehydration and starvation tolerance noted in bed bugs. These gene sequences will facilitate the discovery of other physiological and behavioural mechanisms underlying the extreme dehydration and starvation tolerance of bed bugs.

The bed bug genome shows strong candidates for the key members of the Toll, Imd and Jak/STAT immune pathways, although the *C. lectularius* repertoire is arguably more sparse for those pathways than in holometabolous insects[41]. Recognition proteins are under-represented, as are antimicrobial peptides (two recognition proteins and two defensins and a cluster of three diptericin-like peptides; Supplementary Data 29), although the latter are notoriously difficult to identify by sequence similarity. The RNA interference pathway is represented in the *C. lectularius*

genome with multiple paralogues for Dicer, Argonaute and other enzymes required for this defence pathway.

Symbiosis and lateral gene transfer. Obligate hematophagy can result in significant deficiencies in specific micronutrients that are poorly represented in blood. *Wolbachia*, a common endoparasite that can affect growth and reproduction in many insect species[42], has evolved a symbiotic nutritional relationship with *C. lectularius*[8,9]. *Wolbachia* provides the bed bug with a cocktail of specific B vitamins that are critical for reproduction and development[8,9]. We annotated genes associated with B vitamin metabolism and determined, as with other insects, that bed bugs possess the genes necessary for B vitamin salvage and conversion after their ingestion in blood or synthesis by *Wolbachia* (Supplementary Note 19).

A computational pipeline[43] was used to detect bacterial scaffolds within the assembly as well as candidate LGTs from bacteria to the bed bug. The nearly complete *Wolbachia* endosymbiont of *C. lectularius* was assembled into 16 scaffolds (Supplementary Data 21). In addition, the nearly complete genome of a *Staphylococcus* associate of bed bugs was assembled into 15 scaffolds, which include three plasmids and a ~3.16-Mb chromosome (Supplementary Data 21). On the basis of high-sequence similarity of chromosomal scaffolds, this bacterium is a close relative of *S. xylosus*, an associate of the skin of humans and other animals[44]. *Staphylococcus* bacteria are commonly found in bed bugs based on two microbiome surveys[12,45], including on the male genitalia and inside the female body, and we report here the first draft genome of this *C. lectularius* associate. There is evidence of sexual transmission of *Staphyloccus*[12]. Further studies are necessary to determine whether this bacterium is routinely acquired from human hosts or is a strain specifically adapted to *Cimex*. A third scaffold was assembled with homology to the bacterium *Pectobacterium carotovorum*. This bacterium is typically associated with plants, and the scaffold is only 250 kb in size, whereas *P. carotovorum* genomes are typically ~5 Mb. Further, the scaffold does not contain a ribosomal locus, and therefore this bacterium is unlikely to be an endosymbiont of *Cimex* with a severely reduced genome size. Given that coverage of this scaffold is similar to the genome coverage (Supplementary Data 21), we speculate that this may be a large lateral gene insertion in *Cimex*; however, further study is needed to resolve this question.

The bed bug shows evidence of extensive bacterial LGTs in its genome. In addition to the case described above, there are 805 candidate LGTs of size >100 bp that appear to be scattered throughout the bed bug genome (Fig. 3). This is the largest number of candidate LGTs found in screening of 14 arthropod genomes using this pipeline. LGTs from the genus *Arsenophonus* ($n=459$ or 57%) are the most commonly found, followed by *Wolbachia* ($n=87$ or 10.9%). Other genera represented include *Sodalis*, *Hamiltoniella* and *Peptoclostridium*. The large number of *Arsenophonus* LGTs found is uncommon in insect genomes so far screened, with numbers typically ranging from 0–22. *Arsenophonus* is a widely distributed arthropod-associated bacterium[46], but has not been reported in *Cimex* and no scaffolds for this bacterium were detected in the genome assembly. The type species *A. nasoniae*[47] has a sequenced genome[48]. It causes male killing in a parasitoid wasp, whereas phenotypic effects of other *Arsenophonus* are less well understood. The second most common source of candidate LGTs in *Cimex* is *Wolbachia*, which is a known mutualistic endosymbiont. *Wolbachia* sequences from this symbiont assembled into a nearly complete genome with high sequence coverage. Because of the presence of *Wolbachia* bacteria in *Cimex*, it is possible that some apparent LGTs are due to assembly errors joining *Wolbachia* and *Cimex* sequences. However, examination of junctions between eukaryotic and prokaryotic sequences for spanning sequence reads and cloned paired ends strongly supports that nearly all of these are legitimate LGT events. In addition, LGT–eukaryotic sequence junctions were amplified for five of six candidates, confirming their presence in the genome (Supplementary Fig. 42). The LGTs found in *Cimex* appear to be unique insertions, as no matches were found to the closest published insect genome (*A. pisum*). Comparative studies among *C. lectularius* populations and related species will be important to determine whether there is genomic variation in LGTs among bed bugs.

The typical pattern of LGT evolution is expected to be insertion (most likely due to non-homologous DNA repair mechanisms) followed by degradation and loss. In this way, LGTs are similar to nuclear mitochondrial DNA insertions found in the genome of most eukaryotes[49]. However, bacterial LGTs can also evolve into functional eukaryotic genes, providing novel biochemical functions in the eukaryote[50]. Most candidate LGTs in *C. lectularius* show no or only traces of gene expression in RNA-seq data from adult males and females, and are thus unlikely to be functional. An exception is a *Wolbachia* LGT on scaffold 132, which encodes a patatin-like gene. Bacterial patatin-like genes have lypolytic properties and can be involved in pathogenicity of some bacteria[51,52]. This LGT shows high

a

Genus	Candidate LGTs
Arsenophonus	459
Wolbachia	87
Sodalis	50
Hamiltonella	25
Peptoclostridium	14
Methanococcus	9
Serratia	9
Shewanella	9
81 Other genera	143
Total	805

b

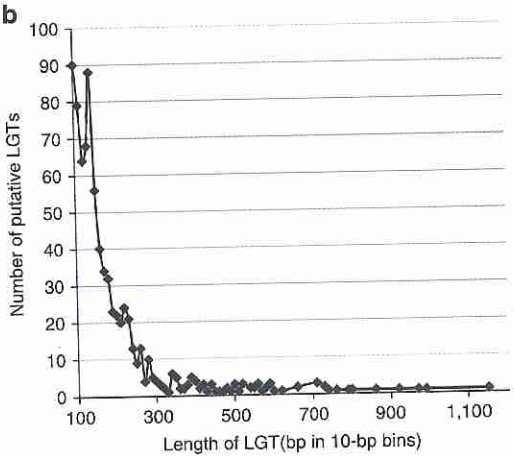

Figure 3 | Summary of putative lateral gene transfers (LGTs) >100 bp in the *C. lectularius* assembly. (**a**) Number of candidate LGTs identified. (**b**) Length of candidate LGTs in bins spanning 10 bp.

expression in adult males but no expression was detected in adult females. While the function and detailed male expression pattern of this gene remain to be determined, we speculate that it may be involved in the unusual insemination mechanism of *Cimex*.

Genes associated with pesticide resistance. A major factor for the increased prevalence of bed bugs in the past two decades, and a contributing factor to the immense difficulties in eradicating infestations, has been the pervasiveness of pyrethroid resistance[4,53–56]. Resistance can result from multiple mechanisms that include target-site mutations, differential gene expression, alterations in the permeability of the cuticle or digestive tract and behavioural changes[4,57–59]. Transcriptomic evidence supports the presence of multiple resistance mechanisms in bed bug populations[4]. To fully understand these potential mechanisms, we manually annotated genes associated with pesticide resistance, including cuticular proteins that can impede pesticide penetration and enzymes that can detoxify pesticides.

V419L and L925I mutations in the voltage-gated sodium channel α-subunit gene have been identified and shown to be responsible for deltamethrin (a pyrethroid) resistance in bed bugs[60]. Molecular analysis of bed bug populations from across the USA and Europe found that >80% and >95% of the respective populations contained V419L and/or L925I mutations in the voltage-gated sodium channel gene, indicating widespread distribution of target-site-based pyrethroid resistance[7,61]. Previous studies showed that higher expression of genes coding for metabolic enzymes including P450s, carboxylesterases and glutathione-*S*-transferases and a reduction in penetration due to higher expression of cuticular protein genes are likely responsible for insecticide resistance of bed bugs[4,59].

Insect genomes code for four distinct clades of P450s called clans: the CYP2, 3, 4 and Mito clan. The *C. lectularius* genome contains 58 genes and one pseudogene coding for P450 enzymes (Supplementary Data 22). Relatively few insect P450s with known or suspected physiological functions are significantly conserved across species[53], and these tend to be involved in biosynthesis of hydrocarbons that cover the insect exoskeleton and prevent desiccation (CYP4G subfamily[4]). Most bed bug P450s (36/58 genes) are members of the highly diverse CYP3 clan; these genes lack clear orthologous relationships and thus are likely involved in species-specific functions. Several transcriptomic analyses have demonstrated substantial overexpression of some bed bug P450s in a manner that was correlated with metabolic resistance to the pyrethroid insecticide deltamethrin[4,54,58]. Four of the P450 genes identified in the *Cimex* genome (CYP397A1, CYP398A1, CYP4CM1 and CYP6DN1) are known to be overexpressed in deltamethrin-resistant populations[4]. Knockdown in the expression of these four P450 genes by RNA interference caused a reduction in deltamethrin resistance levels[4,62]. In addition, RNA interference of cytochrome P450 reductase, which encodes a co-enzyme required for P450 activity, reduced deltamethrin resistance levels in resistant populations of *Cimex*[62]. These results indicate that P450s play an important role in *Cimex* insecticide resistance.

ATP-binding cassette (ABC) transporters play important roles in the shuttling of a wide variety of substrates including hormones, ions, sugars, amino acids, vitamins, peptides, polysaccharides, lipids and insecticides[63]. In a recent study, the expression of 8 out of 27 contigs coding for ABC transporters was elevated in pesticide-resistant *Cimex* populations relative to susceptible populations[54]. We identified 24 additional ABC transporters (Supplementary Data 23); in total, *Cimex* encodes 51 ABC transporters belonging to all eight known classes. Interestingly, 25 of the 51 transporters belong to ABCG/H class, members of which are known to be involved in transport of xenobiotics[63]. Three of the ABC transporters identified in the *Cimex* genome (ABCG20-3, ABCG23-5 and ABCH-B; previously named ABC8, ABC9, ABC10 and ABC11 based on transcriptome analysis[54]) are overexpressed in the epidermis in 21 field-collected resistant populations relative to susceptible populations[4]. In addition, knockdown of ABCG20-3 (ABC8 and ABC9 are encoded by this gene) reduced deltamethrin resistance[4]. With the complete set of ABC transporter genes, future studies will be able to fully assess their contribution to insecticide resistance.

Carboxylesterases are critical in the metabolic breakdown of insecticides[64]. We identified 30 carboxylesterase genes in the *Cimex* genome (Supplementary Data 24). Half of them are located in a single cluster in scaffold 81, suggesting significant gene duplication, and one carboxylesterase, CLE11776 (previously named ClC21331), is expressed at very high levels in most of the 21 field-collected populations tested[4]. We also identified 12 glutathione-*S*-transferase genes in the *Cimex* genome, which was similar to the number identified previously by transcriptome studies[58] (Supplementary Data 25).

The bed bug cuticle plays a substantial role in resistance to insecticides; this is thought to be due (at least in part) to changes in the expression of cuticle proteins in resistant strains[4,54,59]. Using the criteria established by Willis[65], we identified 273 genes that encode putative cuticle proteins (Supplementary Note 6). Of these, 169 genes could be placed in one of eight families (CPR, CPRL, CPF, CPFL, CPAP1, CPAP3, TWD and Dumpy), with an additional 104 proteins consisting of repeated low-complexity sequences (AAPV/GGY) commonly associated with cuticle proteins but without a defining conserved domain (Supplementary Data 10). Approximately, 70% of bed bug cuticle protein genes were arranged in clusters ranging from 3 to 19 genes (Supplementary Data 11; Supplementary Fig. 6); clusters were largely type specific and emphasize the potential for regulatory changes that might influence the expression of the entire cluster.

As in other insects, the CPR family represents the largest single family of putative cuticle protein genes found in the bed bug genome. The 121 CPR-type genes we identified (Supplementary Fig. 7) are slightly more than in *Drosophila*[66] but fewer than in the silkworm *Bombyx mori*[67] or the malaria mosquito *Anopheles gambiae*[68]. We note a bed bug-specific expansion in this family consisting of a novel 10 gene cluster whose members encode two chitin-binding domains each; similar gene structures were not identified in the pea aphid or any of the dipteran genomes.

Traumatic insemination. Among the >40 independent evolutionary events in different lineages leading to traumatic mating, bed bugs are among the best-studied cases[69]. Females evolved a novel organ that reduces the physical trauma of copulation by means of a dense aggregation of the super-elastic protein resilin[70]. Intriguingly, our genome analysis revealed a recent expansion in pro-resilin genes, with 13 such genes containing the pro-resilin characteristics of a chitin-binding domain and consisting of >20% glycine. The pro-resilin gene CPR57 is over 600 amino acids with >40% glycine (Supplementary Figs 7,8). A similar diversification of the resilin gene family is not seen in the related pea aphid (six genes even though there are a similar number of CPR-type cuticle proteins, ~115 (refs 65,66)) nor is it seen in other blood-sucking insects that experience the enormous stretching of the cuticle to accommodate the blood meal (*Aedes*, *Pediculus*, and *Anopheles*; 2–6 genes), indicating lineage-specific adaptive significance of the resilin gene family.

Conclusions. The sequencing, assembly, annotation and manual analyses of the *C. lectularius* genome provide an important and timely resource for understanding the biology of this human

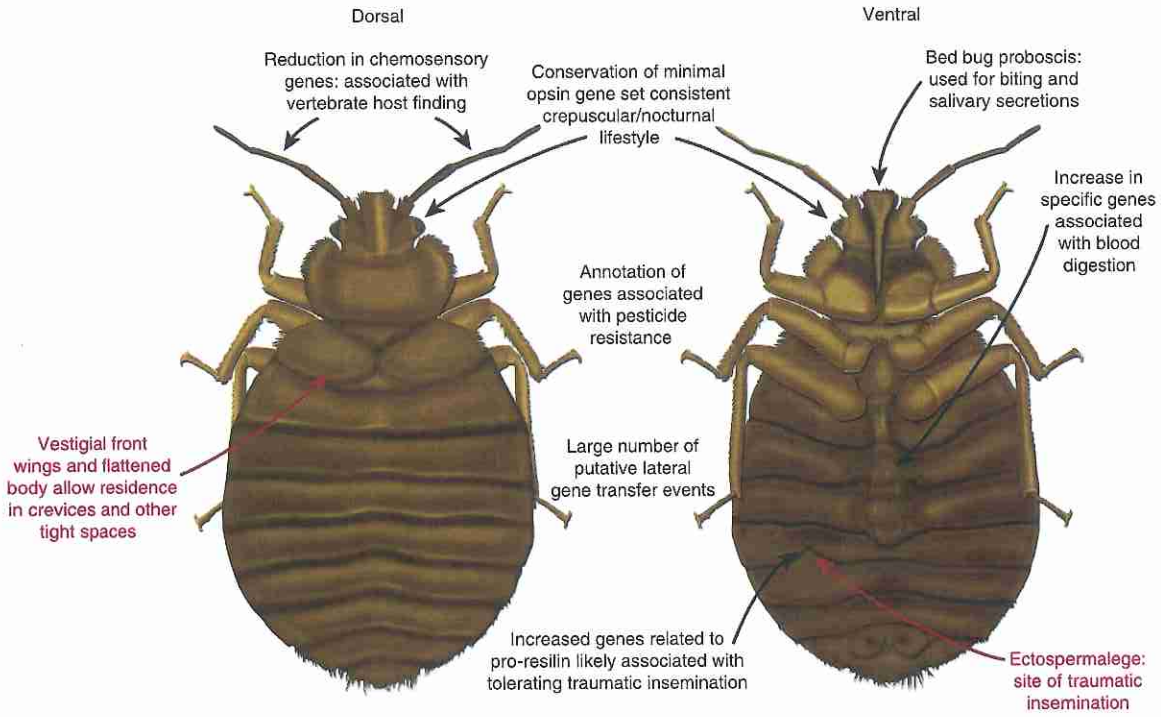

Figure 4 | Synopsis of the contributions from the *C. lectularius* genome to understanding key biological processes. Red, general characteristics of bed bugs; black, key aspects identified and expanded by genome sequencing and manual curation.

ectoparasite, as summarized in Fig. 4. It also will serve as a gateway for the discovery of new targets for control of bed bug populations. This reference genome sequence is of a bed bug strain that is common in laboratory cultures and collected before the introduction of pyrethroid insecticides. What triggered the current bed bug resurgence, and did bed bugs originate from one or multiple sources? This genome sequence will facilitate the discovery of molecular markers and single-nucleotide polymorphisms that will enable research to address these questions. There are many related *Cimex* species that specialize on non-human vertebrate hosts. Comparative genomic studies should reveal specific chemosensory and digestive specializations that define anthropophagy in *C. lectularius*. Even host-associated differentiation within this species requires further genomic studies to understand why one lineage of *C. lectularius* prefers humans and another lineage prefers bats, and how the two remain genetically differentiated even within the same home.

Traumatic insemination has evolved multiple times in various unrelated taxa. The sequenced bed bug genome will serve as an important resource for studies on male-expressed gene networks that ensure sperm transfer despite the female's immune response and other female-expressed pathways that may facilitate cryptic choice of mates. Most haematophagous arthropods have been implicated as vectors of human or animal pathogens, but bed bugs have not. Pathogenic organisms have been isolated from bed bugs, and bed bugs have been shown experimentally to be competent vectors, for example, of American trypanosomes. However, no evidence exists of disease transmission by bed bugs in the field. The sequenced genome will enable studies on mechanisms that actively hinder or do not support vertebrate pathogen survival, proliferation and transmission in bed bugs. Finally, allergenic proteins excreted by anthropophilic arthropods (for example, cockroaches and house dust mites) tend to serve as aetiologic agents of human allergic disease and asthma. Bed bug infestations reach densities of thousands of individuals per home, which may generate high levels of specific antigens. The sequenced genome will provide a platform for the identification and characterization of bed bug-produced allergens that may negatively affect the health and well-being of those whose economic status, unfortunately and almost certainly, ensures that humans and bed bugs will remain closely associated for the foreseeable future.

Methods

Bed bug rearing and RNA/DNA extraction. The bed bug colony was originally established from bed bugs collected in Fort Dix, New Jersey in 1973 and maintained by Dr Harold Harlan (hence Ft. Dix = Harlan strain). This strain is susceptible to all insecticides and has served as a reference strain for pesticide resistance assays, transcriptomic studies and many basic physiological and behavioural studies. The colony was maintained at 27 °C, 50 ± 5% RH and a photoperiod of 12:12 (L:D). Insects were fed in the laboratory through a parafilm-membrane feeder with defibrinated rabbit blood heated to 37 °C by a circulating water bath. Bed bugs were prepared for DNA extraction and sequencing by passing them through six generations of full-sib mating. Only a single sibling pair at each successive generation was used to parent the next generation. This inbred line, now in its 23rd generation, is available upon request (coby@ncsu.edu).

Genomic DNA was isolated from individual female adults using DNeasy Blood & Tissue Kit (Qiagen Inc, Valencia CA). Total RNA was isolated from three females and three adult males separately using the TRI reagent (Molecular Research Center Inc., Cincinnati, OH). The RNA was treated with DNase I (Ambion Inc., Austin, TX). The residual DNase I was removed using resin (Ambion Inc., Austin, TX).

Genome sequencing and assembly. The bed bug is 1 of 30 arthropod species sequenced as a part of the pilot project for the i5K arthropod genomes project at the Baylor College of Medicine Human Genome Sequencing Center. For all of these species, an enhanced Illumina-ALLPATHS-LG sequencing and assembly strategy enabled multiple species to be approached in parallel at reduced costs. For *Cimex lectularius*, we sequenced four libraries of nominal insert sizes 180, 500, 3 and 8 kb at genome coverages of × 62.4, × 77.9, × 44.42 and × 21.21, respectively (based upon the 650.47-Mb genome size of the assembled genome). These raw sequences have been deposited in the National Center for Biotechnology Information (NCBI) SRA: SRS580017, BioSamples ID: SAMN02649412 and SAMN02434893.

To prepare the 180- and 500-bp libraries, we used a gel-cut paired-end library protocol. Briefly, 1 μg of the DNA was sheared using a Covaris S-2 system (Covaris, Inc. Woburn, MA) using the 180- or 500-bp program. Sheared DNA fragments were purified with Agencourt AMPure XP beads, end repaired, dA tailed and ligated to Illumina universal adaptors. After adapter ligation, DNA fragments were further size selected by agarose gel and PCR amplified for six to eight cycles using Illumina P1 and Index primer pair and Phusion High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA). The final library was purified using Agencourt AMPure XP beads and quality assessed by Agilent Bioanalyzer 2100 (DNA 7500 kit, Agilent Technologies) to determine library quantity and fragment size distribution before sequencing.

Long mate-pair libraries with 3- and 8-kb insert sizes were constructed according to the manufacturer's protocol (Mate Pair Library v2 Sample Preparation Guide art # 15001464 Rev. A PILOT RELEASE). Briefly, 5 (for 2 and 3-kb gap size library) or 10 μg (8–10-kb gap size library) of genomic DNA was sheared to desired size fragments by Hydroshear (Digilab, Marlborough, MA), then end repaired and biotinylated. Fragment sizes between 1.8 and 2.5 kb (2 kb), 3 and 3.7 kb (3 kb) or 8 and 10 kb (8 kb) were purified from 1% low-melting agarose gel and then circularized by blunt-end ligation. These size-selected circular DNA fragments were then sheared to 400 bp (Covaris S-2), purified using Dynabeads M-280 Streptavidin Magnetic Beads, end repaired, dA tailed and ligated to Illumina PE sequencing adaptors. DNA fragments with adaptor molecules on both ends were amplified for 12–15 cycles with Illumina P1 and Index primers. Amplified DNA fragments were purified with Agencourt AMPure XP beads. Quantification and size distribution of the final library was determined before sequencing as described above.

Sequencing was performed on Illumina HiSeq2000s generating 100-bp paired-end reads. Reads were assembled using ALLPATHS-LG (v35218, http://www.broadinstitute.org/software/allpaths-lg/blog/) and further scaffolded and gap-filled using in-house tools Atlas-Link (v.1.0) and Atlas gap-fill (v.2.2) (https://www.hgsc.bcm.edu/software/). This yielded an assembly of size 650.47 Mb with contig N50 of 23.5 kb and scaffold n50 of 7.17 Mb. The assembly has been deposited in the NCBI: BioProject PRJNA167477.

Automated gene annotation using a Maker 2.0 pipeline adapted for arthropods. The bed bug is 1 of 30 i5K pilot genome assemblies that were subjected to automatic gene annotation using a Maker 2.0 (http://www.yandell-lab.org/software/maker.html) annotation pipeline tuned specifically for arthropods. The pipeline is designed to be systematic, providing a single consistent procedure for the species in the pilot study, scalable to handle 100s of genome assemblies, evidence guided using both protein and RNA-seq evidence to guide gene models and targeted to utilize extant information on arthropod gene sets. The core of the pipeline was a Maker 2 instance, modified slightly to enable efficient running on our computational resources. The genome assembly was first subjected to *de novo* repeat prediction and CEGMA analysis (http://korflab.ucdavis.edu/datasets/cegma/) to generate gene models for initial training of the *ab initio* gene predictors (Supplementary Data 33). Three rounds of training of the Augustus (http://bioinf.uni-greifswald.de/augustus/) and SNAP (http://korflab.ucdavis.edu/software.html) gene predictors within Maker were used to bootstrap to a high-quality training set. Input protein data included 1 million peptides from a non-redundant (nr) reduction (90% identity) of Uniprot Ecdysozoa (1.25 million peptides) supplemented with proteomes from 18 additional species (*Strigamia maritima, Tetranychus urticae, Caenorhabditis elegans, Loa loa, Trichoplax adhaerens, Amphimedon queenslandica, Strongylocentrotus purpuratus, Nematostella vectensis, Branchiostoma floridae, Ciona intestinalis, Ciona savignyi, Homo sapiens, Mus musculus, Capitella teleta, Helobdella robusta, Crassostrea gigas, Lottia gigantea* and *Schistosoma mansoni*) leading to a final nr peptide evidence set of 1.03 million peptides. RNA-seq from *C. lectularius* adult males and females was used judiciously to identify exon–intron boundaries but with a heuristic script to identify and split erroneously joined gene models. We used CEGMA models for QC purposes: for *C. lectularius*, of 1,977 CEGMA single-copy orthologue gene models, 1,928 were found in the assembly, and 1,892 in the final predicted gene set. Finally, the pipeline uses a nine-way homology prediction with human, *Drosophila* and *C. elegans*, and InterPro Scan5 to allocate gene names. The automated gene set is available from the BCM-HGSC website (https://www.hgsc.bcm.edu/arthropods/bed-bug-genome-project) and at the National Agricultural Library (https://i5k.nal.usda.gov).

Community curation of the bed bug genome. Thirty-two groups were recruited through the i5k pilot project to manually curate the MAKER-predicted gene set Clec_v0.5.3. These curators selected genes or gene families based on their own research interests. Manual curation occurred via the Web Apollo software, a web-based graphical user interface for gene model curation that allows curators to create and view changes to gene models in real time. A *C. lectularius* Web Apollo (http://genomearchitect.org/) instance was made available (https://apollo.nal.usda.gov/cimlec/jbrowse/) to display evidence included in the generation of the Clec_v0.5.3 gene predictions. This Web Apollo instance also incorporates aligned RNA-seq and transcriptome data sets provided to the scientific community by Zach Adelman (Virginia Tech) that were not included in the MAKER analysis. Curators were provided a webinar-based training session on the Web Apollo software (courtesy of Monica Muñoz-Torres, Lawrence Berkeley National Laboratory) and were asked to adhere to a set of curation rules (https://i5k.nal.usda.gov/content/rules-web-apollo-annotation-i5k-pilot-project). After the curation period, the manually curated models were exported in gff3 format and quality checked for formatting and curation errors, and then integrated with the MAKER-predicted gene models to generate a non-redundant official gene set OGSv1.1. A subsequent quality-control check using two separate gff3-checking pipelines (https://github.com/hotdogee/gff3-py/releases/tag/0.3.0; https://github.com/chienyuehlee/gff-cmp-cat) and custom scripts resulted in an updated OGSv1.2.

Orthology/phylogeny analyses. The OrthoDB (http://orthodb.org/) resource was used to find shared orthologues among *C. lectularius* (v1.0) and seven other arthropods: *Daphnia pulex, Pediculus humanus humanus* (= *Pediculus humanus corporis*), *Acyrthosiphon pisum, Apis mellifera, Tribolium castaneum, Danaus plexippus* and *Drosophila melanogaster*. Custom Perl scripts were used to find the number of genes in each category (Fig. 1, single copy, present in all species and so on). For the phylogenetic analysis, only the single-copy orthologues were used to build a concatenated phylogenetic tree using RAxML (http://sco.h-its.org/exelixis/software.html). Briefly, a multiple sequence alignment was performed using MUSCLE (http://www.drive5.com/muscle/) for each orthologous cluster, separately. Then, the resulting alignments were trimmed using trimAl (http://trimal.cgenomics.org/) and these alignments were concatenated using the 'seqret' program from the EMBOSS suite (http://emboss.sourceforge.net/). This concatenated alignment was used to build the phylogeny using RAxML 7.6.6 with 100 bootstraps.

BUSCO-based quality assessment. The completeness of genome assemblies can be measured by searching for the presence of conserved genes. Absence of such genes means that the assembly is incomplete to a greater or lesser degree depending on the fraction of missing genes. Moreover, if these conserved genes are also single copy, the assembly can also be tested for unexpected duplications, which is a sign of erroneous haplotype assembly. To this end, we used the BUSCO (http://busco.ezlab.org/)[18], to measure the completeness of the bed bug genome as well as its set of predicted genes (Supplementary Data 3). We used the Arthropoda gene set, which consists of 2,675 single-copy genes that are present in at least 90% of Arthropoda.

Lateral gene transfer identification. Putative LGT events in the assembled bed bug genome were computationally identified using two different python-based computational pipelines. The bed bug assembly was first analysed using the homology-based pipeline described in Wheeler *et al.*[43] Because this pipeline outputs data on the best putative LGT candidate on each scaffold (best on e-value), along with the number and range of putative LGTs on the scaffold, it was most helpful for identifying scaffolds that appear to be of bacterial, not bed bug, origin (Supplementary Data 21). In addition, if the best putative LGT on each scaffold had a higher bacterial score than animal score, it was manually annotated using Blastn and Blastx analysis to the NCBI nr/nt database. In addition, 12 scaffolds with a high number of potential LGTS were manually searched for additional LGT candidates using Blastn similarity (e-value 1e − 5 cutoff) on the NCBI nr/nt database.

Due to the vast number of putative LGT candidates, a second python script was used to break long scaffolds into 1,000-bp intervals and search each of them against the bacterial database. Any positive hits of the 1,000-bp regions were then searched against the animal database. The bacterial database contained ~1,000 bacterial species and was masked for low-complexity regions using the NCBI Dustmasker function. The animal database contained transcripts from a representative from each of the following animal genera: *Anopheles, Apis, Drosophila, Xenopus, Tribolium, Nasonia, Daphnia, Strongylocentrotus, Mus, Homo sapiens, Aplysia, Caenorhabditis, Hydra, Monosiga* and *Acanthamoeba*. The significance e-value cutoff used was 1e − 5 for both the animal and bacterial hits. Regions of bacterial similarity that fell from the end of one 1,000-bp interval to the adjacent interval were joined if they were <50-bp apart. Putative LGT regions that were ≥100 bp were used in the final analysis. As a further computational confirmation, junctions between candidate LGTs and flanking eukaryotic sequences were confirmed using mate-pair data from the different insert size libraries.

Note added in proof: Since this manuscript was submitted the genome of the closely related hematophagous hemipteran, *Rhodnius prolixus*, was published (Mesquita *et al.* Genome of *Rhodnius prolixus*, an insect vector of Chagas disease, reveals unique adaptations to hematophagy and parasite infection. *Proc. Natl Acad. Sci.* USA (early edition) doi/10.1073/pnas.1506226112) and has not been included in analyses associated with this study.

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Author contributions

Cob. S., S.R. and S.R.P. conceived the project. Cob. S., J.B.B., S.R.P. and S.R. managed and coordinated the project. Cob. S., W.B. and E.L.V. provided biological materials through full-sib mating. S.R.P. and F.Z. performed DNA and RNA extraction. S.D., S.R., Hu.D., Y.H., Ha.D., D.M.M. and R.A.G. managed library preparations and sequencing. S.R. S.L.L. and H.C. constructed DNA/RNA libraries and performed sequencing. S.R., D.S.T.H., S.C.M., J.Q. and K.C.W. performed the genome assembly and gene prediction. M.P., C.C., C.-Y.L., H.L. and D.S.T.H. developed and implemented Web Apollo manual curation. R.M.W., E.M.Z. and P.I. performed orthology and phylogenetic analyses. J.B.B., A.J.R., E.M.S., R.W.H., G.M.A., S.R.P., H.G., J.N.S., M.M., D.R.N., Z.N.A., C.D., C.W., V.R., S.W., P.M., N.P., Wo.B., R.P., K.R., P.J., R.N., Cor. S., M.-C.O., F.M., A.D.G., Mi. E.S., Ma.E.S., B.F.P., K.R.R., H.M.R., B.A.H., D.A., A.J.J.C., P.N.R., Em.S., Es.S., S.V., A.K., S.-J.A., E.J.D., J.H.W., J.M.H., A.D., O.M., C.Z., M.F., M.T.W., J.W.J., K.A.P., I.M.V.J., J.-J.Z., J.D.E. and J.M.C.R. participated in manual curation and contribution of Supplement Notes. J.S.J. analysed genome size. J.H.W., A.D. and D.W. performed bacterial scaffold detection and LGT analyses. M.A.E.A. performed confirmation experiments for selected LGTs. Cob. S., S.R.P., J.B.B., Z.N.A., K.R., J.H.W., D.W. and S.R. wrote the manuscript. J.B.B., A.J.R., E.M.S., J.M.H. and E.C.J. organized Supplementary Materials. J.B.B., S.R. and G.M.A. prepared figures for the manuscript. All authors commented on the manuscript.

Additional information

Accession codes: Data for the *Cimex lectularius* genome has been deposited in the GenBank/EMBL/DDBJ Bioproject database under the accession code PRJNA167477. Raw genomic sequence data is deposited in the GenBank/EMBL/DDBJ sequence read archive under the accession codes SRX498126, SRX498127, SRX498128, and SRX498129. The genome assembly has been deposited in GenBank under the accession code GCA_000648675.1. RNA-seq datasets used in gene prediction have been deposited to the in GenBank/EMBL/DDBJ sequence read archive under the accession codes SRX906994 and SRX907005.

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Current Biology
Report

Bedbugs Evolved before Their Bat Hosts and Did Not Co-speciate with Ancient Humans

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SUMMARY

All 100+ bedbug species (Cimicidae) are obligate blood-sucking parasites [1, 2]. In general, blood sucking (hematophagy) is thought to have evolved in generalist feeders adventitiously taking blood meals [3, 4], but those cimicid taxa currently considered ancestral are putative host specialists [1, 5]. Bats are believed to be the ancestral hosts of cimicids [1], but a cimicid fossil [6] predates the oldest known bat fossil [7] by >30 million years (Ma). The bedbugs that parasitize humans [1, 8] are host generalists, so their evolution from specialist ancestors is incompatible with the "resource efficiency" hypothesis and only partially consistent with the "oscillation" hypothesis [9–16]. Because quantifying host shift frequencies of hematophagous specialists and generalists may help to predict host associations when vertebrate ranges expand by climate change [17], livestock, and pet trade in general and because of the previously proposed role of human pre-history in parasite speciation [18–20], we constructed a fossil-dated, molecular phylogeny of the Cimicidae. This phylogeny places ancestral Cimicidae to 115 mya as hematophagous specialists with lineages that later frequently populated bat and bird lineages. We also found that the clades, including the two major current urban pests, *Cimex lectularius* and *C. hemipterus*, separated 47 mya, rejecting the notion that the evolutionary trajectories of *Homo* caused their divergence [18–21].

RESULTS AND DISCUSSION

The Molecular Phylogeny

The consensus tree (Figure 1) of five DNA sequence segments of four genes (1) shows that the Cimicidae are monophyletic and firmly placed within the Cimicomorpha [4, 22–25]; (2) provides robust resolutions of other debated relationships (Figure 1), including the paraphyly of the groups that parasitize swallows (martin bugs, previously genus *Oeciacus*) [21]; and robustly identifies *Primicimex*+*Bucimex* (3) as a monophyletic group (Figure 1) supporting morphological arguments [1] and a concurrent investigation [26], and (4) as the sister of the remaining extant Cimicidae, solving a long-standing problem in insect systematics [22–25, 27, 28]. (5) The biogeographical distribution (Figure 1) shows continent-restricted ranges of higher cimicid taxa that may relate to either occasional cross-continent host dispersal or geological events. Consistent with the latter, ancestral clades (Primicimicinae and Afrocimicinae) are restricted to continents that developed from western Gondwana [29]. India may not have been colonized until about until 75 mya (Cacodminae), when it started to collide with Asia, and

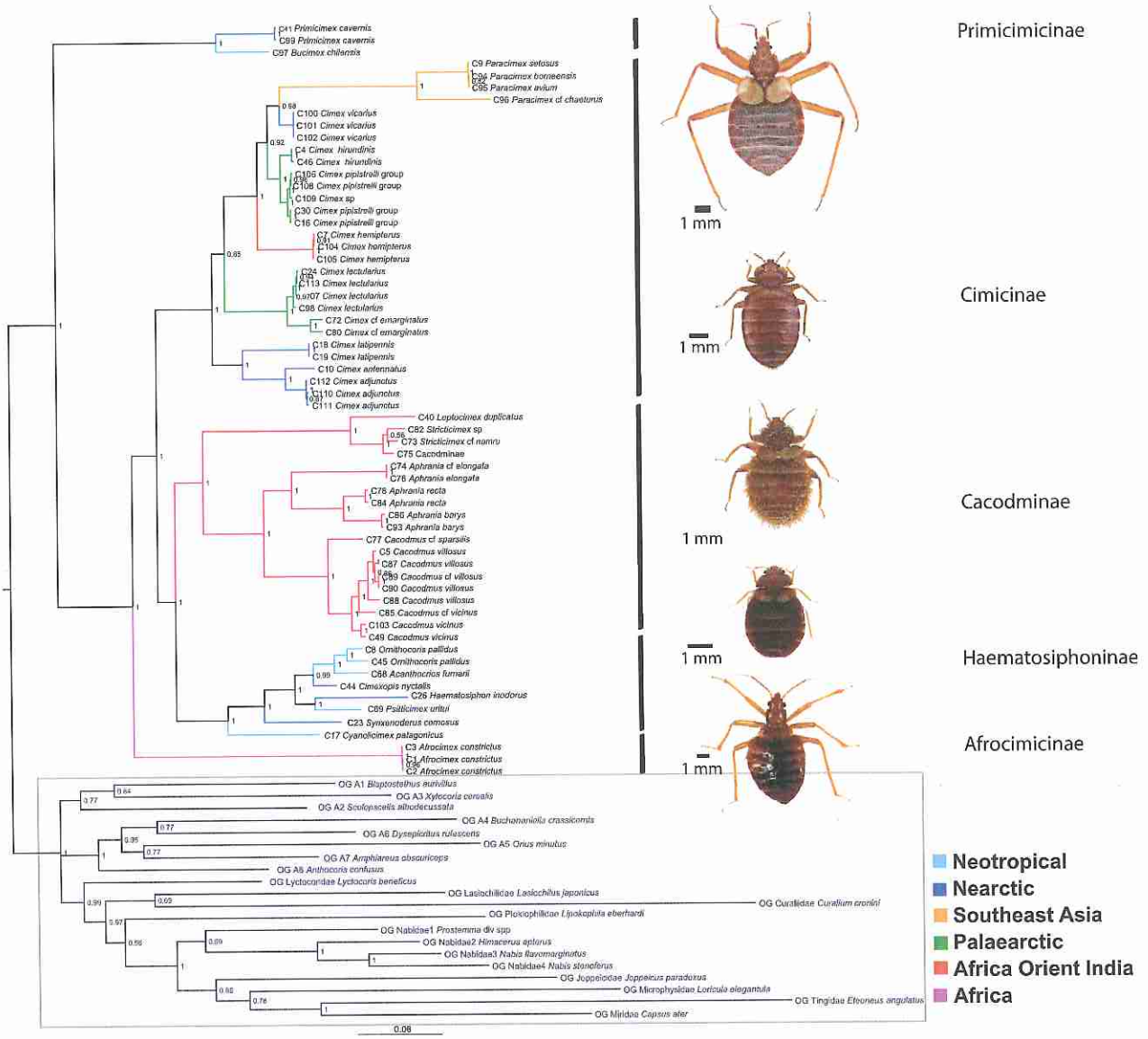

Figure 1. Phylogeny of the Bedbug Family (Cimicidae)

Bayesian consensus tree based on five gene sequences showing the biogeographical distribution (branch colors) and classical taxonomy at the subfamily level. Photographs show morphologically typical representatives of each subfamily. Numbers beside the nodes indicate posterior probability values. The branch lengths scale represents the number of estimated nucleotide substitutions per site. For species, sample codes, collection details, and sequences of outgroups (boxed in shaded gray and taken from GenBank), see Table S1.

Europe not before 50 mya (Cimicinae), following the collision with the African plate. Wallace's line represents a distribution border. Consistent with dispersal by hosts is some degree of host conservatism (see results below) and a (near) cosmopolitan distribution only for human-associated species. On the other hand, it is striking that a continuous form of cross-continent dispersal by hosts has not altered the biogeographic distribution of those cimicids that parasitize swallow species: neither the North American *C. vicarius* nor the European *C. hirundinis* have been recorded in the winter grounds of their hosts, South America and Sub-Saharan Africa, respectively.

Enigmatic Ancestral Host and Multiple Colonization Events of Bats

Independently dating the phylogenetic tree using a fossil from the related family Vetanthocoridae (152 mya) [25] rejects the widely held view [1] that the Cimicidae evolved on bat hosts. Our mean estimate of 115 mya (74–170; 95% highest posterior density [HPD] interval) for the stem of the Cimicidae supports the idea of a minimum age of the group of 100 mya based on fossil evidence [6]. The origin of the Cimicidae crown group with a mean of 93.8 (56–137; 95% HPD) mya is placed 30–50 Ma before the earliest known bats [7, 27, 28, 30] (Figure 2) and 20 Ma before the earliest inferred bats (73 [64–81; 95%] mya) [30]. Our

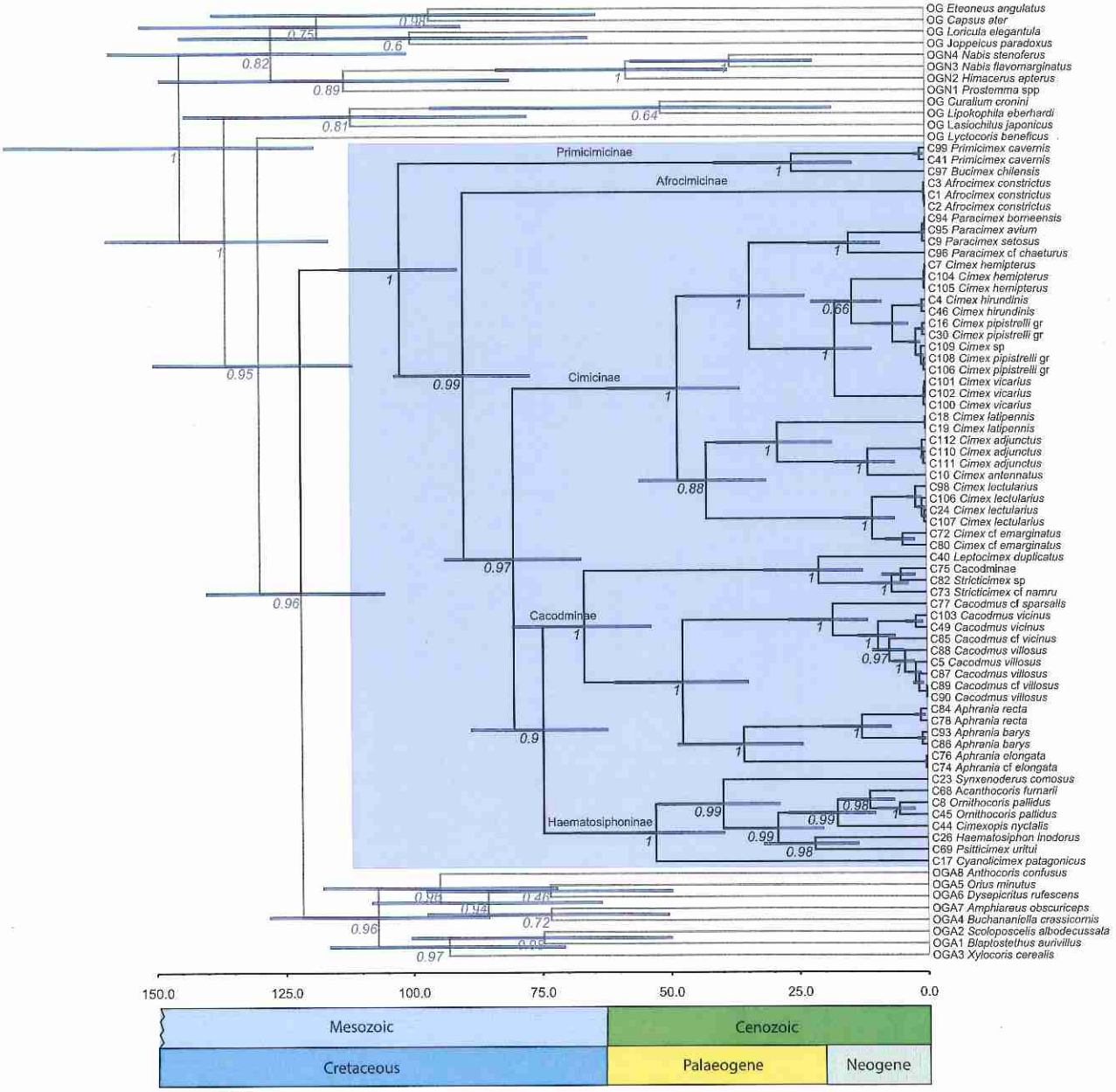

Figure 2. Chronogram of the Bedbug Family (Cimicidae)

Bayesian consensus tree of the Cimicidae and selected outgroup taxa in relation to geological age (mya; x axis). A relaxed clock model [31] was used to date the tree based on two calibration points, fossil Vetanthocoridae (152 mya) [25] and the oldest known fossil cimicid (100 mya) [6]. Numbers below nodes represent Bayesian posterior probability values; blue bars represent 95% highest posterior density intervals of the time estimates in mya. Scale is in millions of years. The Cimicidae are boxed in shaded blue. "gr." stands for group, a taxonomic aggregate. The time estimates returned a mean age of 103 Ma for the crown group of the Cimicidae and are robust against alternate taxonomic assumptions of the Vetanthocoridae (https://doi.org/10.5281/zenodo.2642215).

estimate appears robust: employing the oldest known cimicid fossil as an additional calibration point places the stem species at 122 mya (111–150 mya; 95% HPD; relaxed molecular clock estimation of lineage divergence points within the family) and the crown divergence at 102 mya (91–114 mya; 95% HPD; Figure 2). Our estimate is also robust against previous suggestions that the Vetanthocoridae might be the sister group to all Cimicoidea [32]. Using the Cimicoidea + Nabidoidea divergence as calibration point produces very similar results, dating the Cimicidae ancestor to 127 mya and the first divergence of the crown group to 103 mya. However, the latter approach devaluated the support for some of the clades that were well supported from unconstrained phylogenetic estimates, and the topological constraint for the calibration changed the position of *Afrocimex* (but also with low support values; https://doi.org/10.5281/zenodo.2642215).

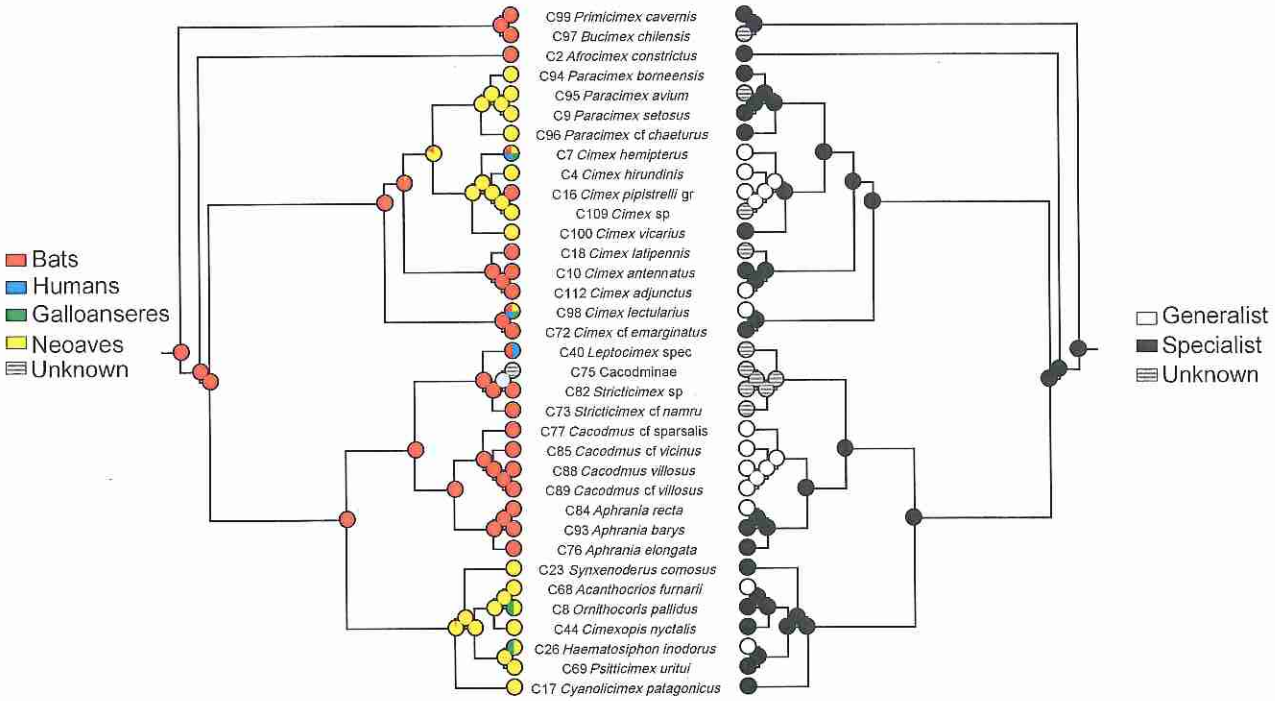

Figure 3. Reconstruction of the Ancestral Hosts of the Cimicidae

Mirror trees showing (A) systematic host groups and (B) their classification as putative host specialist or generalist (see main text for classification; Figures S1 and S2). In (A), colors indicate different host types reported [1, 33–35] (Table S1). In (B), putatively specialized (black) or generalist (white) host uses were reconstructed with (unordered) parsimony. Separate analyses with MrBayes [36] confirmed specialized host use as the ancestral state for all Cimicidae. The result did not change if the two lineages with the highest uncertainty about their ancestral state, i.e., Cimicinae and Cacodminae + Haematosiphoninae were analyzed separately by setting all other clades to an unknown state of G or S (probabilities of bats as ancestral host 98% and ancestral specialist 85% for the Cimicinae and 96% and 98%, respectively, for the Cacodminae + Haematosophinae). *Leptocimex duplicatus* was analyzed as *Leptocimex* spec. to demonstrate human host use in this genus. Results were identical if ancestral analysis host and specialization employed bats or bats + human. The results also did not change if classes of the exact number of currently known host genera were employed (see also Figures S1 and S2). Comparing host and parasite mean ages shows that diversification is not generally driven by co-speciation with hosts on bat or bird hosts; see also Figures S1–S3 and Table S1.

All four ancient bedbug lineages predate the evolution of bats (Figure 2) but were reconstructed to ancestral bat hosts (Figure 3A). This suggests that bats were colonized several times independently, unless the evolutionary origin of bats [7, 27, 28, 30] has been grossly underestimated.

Thus, the stem species of bedbugs evolved 115–122 mya, well before the Cretaceous–Tertiary (K-T) mass extinction boundary, a key event in vertebrate diversification. The identity of the ancestral host(s) from which bats were colonized repeatedly is unknown.

Evolution of Hematophagy

Our phylogeny does not support ancestral host generalism (G) in cimicids (Figure 3B), so we propose the commonly assumed evolution of hematophagy from facultative blood feeding by ancestral predators [3, 4] did not occur. This result is robust against variation in the definition of species along the host specialist (S) and host G axis, depending on the specialization metrics or recording intensity [9–16]. For example, technically, all specialists are "putative specialists" until additional hosts may eventually be found. In any case, the derived state of G holds true if the number of currently known host genera is used (Figure S2) and if G are defined by the phylogenetic distance of their hosts [16], i.e., as using more than one of the four major, phylogenetically deeply diverged host groups of waterfowl (Galloanseres) and other birds (Neoaves), as well as bats (Chiroptera) and humans (Figure 3A). It also holds true for a definition of G accounting for variability within taxonomic groups [16] as being those parasites recorded from more than three host genera (Figure S1). Therefore, hematophagy likely evolved within the true bugs (Heteroptera), in insects that were already specialists and gave rise to the Cimicidae. This result is compatible with the view that the specialist blood-sucking Polyctenidae is the sister group of the Cimicidae [4, 22].

Pattern of Host Shifts

Of the 29 species on our tree that allow a classification, most (24/29; 83%) are S (broadly defined; Figure 3A) or 55% (15/27), using tighter definitions (Figures 3B and S2). Five cimicid species on our molecular tree are G (broadly defined) [1].

Host shifts between bat taxa were common by ancient specialists because most extant bat-parasitic cimicid lineages evolved before their extant hosts' lineages. For example, comparing means of the phylogenetic age of host and parasite (95% lower–upper highest posterior distribution inferred by BEAST) [31], we found that *Afrocimex* (103–77) evolved 67 Ma before its current host *Roussettus* (26–18), *Bucimex*

(42–13) 6 Ma before *Myotis* (25–16), *Primicimex* (42–13) 4 Ma before Tadarida (27–17), or the Cimicinae + Cacodminae + Haematosiphoninae clade (94–65) 26 Ma before the Vespertilionidae (60–50; Figure 3). Host switches from bats to birds also occurred; we identified at least three such independent events (Figure 3A). For bird hosts, the Haematosiphoninae diverged around 50 mya, the bird-parasitic *Paracimex* (around 15 mya) or *Cimex vicarius* (around 18 mya)—long after their respective swift or swallow host groups had appeared in the early Eocene (Figures 2 and 3).

Our host reconstruction indicates that parasite diversification is not generally driven by co-speciation with hosts [14, 15] (but see [37]) for either bat or bird hosts (Figure 3). Together, these observations suggest that the extant pattern of G/S distribution in cimicids is the result of evolutionarily dynamic host transitions.

When examining host transitions at all 31 subterminal nodes on our tree that are classifiable as G or S, we found the highest number (9/31 or 29%) involved host specialists switching host but staying specialist (S→S). Two nodes were G→S transitions (6%), and five (16%) were S→G transitions (or 7/31 [23%] if specialists are defined more strictly; Figures 3B and S2).

The paucity of G→S transitions departs from the general pattern in mammalian parasites [16] and indicates that the "resource efficiency" hypothesis (where host S evolve from G by fitness advantages on specific hosts) [9–11] does not appear to apply to cimicids. An extension of this idea, the "oscillation" hypothesis, proposes that genetic variation or phenotypic plasticity maintained in S species allows them to add hosts to their portfolio (and so become G again), depending on ecological opportunities [12, 14, 16]. Although this hypothesis allows for any number of S and G transitions, S→G transitions should be evenly distributed across evolutionary time if they are regularly oscillating. This prediction was rejected: all seven S→G transitions occurred in a short period, between 10 and 20 mya (cf. Figures 2 and 3).

Acceptance of unusual hosts under ecological opportunities (such as laboratory-forced host feeding) can serve as an indicator of plasticity or genetic variation in host preference [14]. Such propensity to switch hosts has only been recorded in G (Figures 3 and S2), but not in S species [5] (K.R., R.N., and M.T.S.-J., unpublished data; O.B., unpublished data; S.R., unpublished data)—which the oscillation hypothesis requires—but few experimental tests exist. Anecdotal acceptances of unusual hosts outside the laboratory suggested to mimic ecological opportunities created by humans have been reported during guano mining, chicken breeding, or pet keeping, again, however, in G, or unscorable, but not in S species. Unless future systematic screening of such events would reveal a massive usage of unusual hosts by S species, there is little current evidence to suggest that S species commonly oscillate to evolve into G species or that host specialization in the Cimicidae is driven by selection for resource efficiency.

S→S transitions (host switches without extensions in host breadth, or so-called "musical chairs" pattern) [12] are the common pattern in cimicids. The musical chairs hypothesis makes no further predictions [12], but S→S transitions can, like S→G, be based on the ecological opportunities new hosts present [14, 15], such as after major (e.g., intercontinental) dispersal events [16]. In support, for example, two of the three bat-to-bird host shifts in cimicids concerned the Haematosiphoninae and *Paracimex*, where bird hosts replaced bats rather than having been added (Figure 3). Both examples simultaneously involved the colonization of another continent (South America and Southeast Asia). However, other S→S transitions are not related to intercontinental shifts.

The only temporary association of cimicids with the host body would be expected to increase opportunities for alternative host use and hence generalism (such as in mosquitoes). However, the widespread and ancient specialization reported here (predicted for parasites with tight host associations that cannot readily exploit new hosts, such as lice) finds a parallel in selection on salivary proteins [38] and divergence in endosymbionts [39], both of which aid hematophagy.

In conclusion, several bedbug lineages specialized on bats in ancient times, but subsequent host shifts were frequent, and the switches (and expansions of host portfolio) that can be explained by current models of host specialization are related to the ecological opportunities that human activity or intercontinental dispersal provided. As general models of host specialization only had limited ability to predict patterns of host use in cimicids, we examined more specific ideas developed for their colonization of human hosts.

Human Colonization and Ashford's Hypothesis

Three bedbug species routinely use humans as hosts (*C. lectularius*, *C. hemipterus*, and *Leptocimex boueti*) [1, 3, 8], representing three independent events (Figure 3A). All are G, all are recent, and all represent expansions of the host portfolio rather than replacements, i.e., they represent the somewhat more unusual S→G transitions among mammalian parasites [16] (Figure 3). The three colonization events of humans are non-randomly captured by these S→G transitions, which represent just 16% (or broad definition: 23%) of transitions (Fisher's exact test; $p = 0.0022$ [or broad definition of G: $p = 0.0078$]). Thus, humans represent an important, nonrandom target for specialist cimicid species. A fourth possible case of human colonization by *Haematosiphon inodorus*, derived from cultural records [40], would also fall into the S→G category.

Our finding that the *C. hemipterus* and *C. lectularius* lineages diverged ~47 mya clearly rejects Ashford's hypothesis [18], which predicts a divergence that coincides with the split between the *H. sapiens* and the *H. erectus* clades around 1.6 mya. Our results show that *C. lectularius* belongs to a bat-associated lineage and *C. hemipterus* to a bird-parasitic lineage [21] (Figure 3A), so Ashford's idea would require a series of independent host shifts from birds and bats to *Homo* lineages. With one species pair of human parasites showing contrary (lice) [19, 20] and one no support (cimicids) for Ashford's hypothesis, this idea should be rejected or re-tested by dating the split of other species pairs of human parasites.

C. lectularius specifically has also been hypothesized to have colonized humans, or *H. sapiens*, when ancient man started to use caves regularly and so represented a predictable food source [1]. Our analysis shows all clades parasitizing humans had diverged at least 5–10 Ma before the oldest known *Homo* species [41, 42]. The spatial and temporal coexistence of several lineages of hominids [41, 42] allows several transmission scenarios and host shifts. However, because bedbugs are not

known from other extant hominids, or other primates, colonization likely took place in the hominin lineages. Thus, no matter when hominids first entered caves, bat- and bird-parasitizing *C. lectularius* were already there and ready to exploit the new opportunity. Thus, although the fact that bat- and human-associated lineages of *C. lectularius* diverged between 99,000 and 867,000 years ago [43] provides us with a hint of when humans acquired *C. lectularius*, it does not tell us which of the *Homo* lineages or their cave-dwelling behavior was the initial driver for contact. Our work shows that the driver for the association suggested by Ashford is too simplistic.

Conclusions

Our phylogenetic reconstruction shows that bedbugs (Cimicidae) evolved before bats, their previously assumed primary hosts, and colonized them on several subsequent occasions. It supports the view that generalism can evolve when ecological opportunities arise, even after long periods of specialization, and shows that all colonizations of human hosts conform to this view. The phylogeny also served to elucidate debated taxonomic relationships and rejects Ashford hypothesis.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2019.04.048.

A video abstract is available at https://doi.org/10.1016/j.cub.2019.04.048#mmc3.

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AUTHOR CONTRIBUTIONS

S.R., M.T.S.-J., E.H.M., and K.R. designed the study; S.R., O.B., O.D.I., M.T.S.-J., P.B., O.C., E.I.F., M.M., R.N., N.S., E.H.M., F.A.A.K., M.P.L., and K.R. extensively contributed material and sequences; S.R. carried out the molecular work; S.R., E.W., and K.R. analyzed the data; S.R. and K.R. wrote the first draft; and S.R., O.B., M.T.S.-J., M.P.L., E.H.M., E.W., and K.R. carried out the first revision. All authors, except O.D.I., contributed to all subsequent revisions.

DECLARATION OF INTERESTS

R.N. is owner of CimexStore (UK).

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
QIAGEN DNAEasy blood and tissue kit	QIAGEN, Hilden, Germany	(QIAGEN, RRID: SCR_008539)
ExoSAP-IT	Thermo Fisher Scientific	(Thermo Fisher Scientific, RRID: SCR_008452)
BigDye Terminator v3.1 Cycle Sequencing Kit	Thermo Fisher Scientific	(Thermo Fisher Scientific, RRID: SCR_008452)
Full list of primers	Table S2	N/A
Deposited Data		
A list of species analyzed	Table S1	N/A
Outgroup taxa	Table S1	N/A
Age of fossils	[6], [25]	N/A
Host spectrum	[1, 33–35], Table S1	N/A
Bat phylogeny	[27]	N/A
Phylogram of birds	[44]	N/A
Bayesian analysis of individual gene trees (18S rDNA 2 parts, COI, 16S rDNA, 28S D3 rDNA)	this paper	https://doi.org/10.2581/zenodo.2642215
Neighbor-Joining analysis using either strict or relaxed GBlock alignments of all molecular markers separately	this paper	https://doi.org/10.2581/zenodo.2642215
MrBayes consensus tree using one representative species of the closest phylogenetic outgroups	this paper	https://doi.org/10.2581/zenodo.2642215
Maximum Likelihood analysis of the combined molecular dataset.	this paper	https://doi.org/10.2581/zenodo.2642215
Alignment file	this paper	https://doi.org/10.2581/zenodo.2642215
Chronogramme using different a different assumptions of the taxonomic placement of the fossil	this paper	https://doi.org/10.2581/zenodo.2642215
Software and Algorithms		
Sequencher v. 4.5	Gene Codes, Ann Arbor, Michigan	http://www.genecodes.com/ (Sequencher, RRID: SCR_001528)
MrBayes 3.2.1.	[36]	http://mrbayes.sourceforge.net/ (MrBayes, RRID: SCR_012067)
Tracer 1.7	[45]	http://tree.bio.ed.ac.uk/software/tracer/
TreeView (Win32) 1.6.6	[46]	http://en.bio-soft.net/tree/TreeView.html (TreeView, RRID: SCR_013503)
FigTree 1.4.1	[47]	http://tree.bio.ed.ac.uk/ (FigTree, RRID: SCR_008515)
Mesquite 3.2	[48]	https://www.mesquiteproject.org/
RAxML 7.4.2.	[49]	https://sco.h-its.org/exelixis/web/software/raxml/ (RAxML, RRID: SCR_006086)
MEGA v. 6	[50]	http://en.bio-soft.net/tree/MEGA.html (MEGA Software, RRID: SCR_000667)
GBlocks V.0.91b	[51]	http://molevol.cmima.csic.es/castresana/Gblocks_server.html (Gblocks, RRID: SCR_015945)
DAMBE V 5.2.13	[52]	http://dambe.bio.uottawa.ca/DAMBE/dambe.aspx
Beast 1.8.4	[31]	http://www.beast2.org/ (BEAST, RRID: SCR_010228)

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Klaus Reinhardt (klaus.reinhardt@tu-dresden.de).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Sample origin

We obtained material from three main sources over the course of 15 years. First, we contacted the major natural history museums in the world as several species are known only from a single collection from their type locality. However, most of this material dated from the 1960s and 1980s and was too old for our analysis, museum material from only two species could be used, material for one of which was subsequently obtained otherwise. Second, between 2002 and 2015, we contacted researchers with requests for specimens. Researchers working on cimicids provided material from 10 species. We also contacted approximately 500 researchers that work in caves, on cave-dwelling bats or other putative bedbug hosts, such as swallows and swiftlets. Approximately half the people responded, and about 30 respondents promised to send material. From those who did send material, an extra 18 species were obtained. Third, between 2000 and 2014, the authors undertook field trips to obtain material, adding 10 species. This resulted in a total of 38 species, of which 34 species from 62 localities yielded sufficient DNA for the analysis (Table S1). Unfortunately, existing *Latrocimex* material from Brazil [53] or its sequences were not at our disposal to be analyzed.

Taxon sampling

In total, 34 species of Cimicidae were analyzed, representing 17 genera from 5 out of 6 currently recognized subfamilies [1]. The most closely related families were chosen as outgroups: Nabidae, Anthocoridae, Plokiophilidae, Microphysidae, Curaliidae, and Joppeicidae [4, 23–25], except the Polyctenidae (for which we obtained no material). We also included representatives of two more distant outgroups, the Tingidae and Miridae. All outgroup taxa sequences were obtained from GenBank (Table S1).

METHOD DETAILS

DNA extraction, PCR amplification, and DNA sequencing

Nuclear and mitochondrial genomic DNA was extracted from 70%–96% ethanol-preserved specimens using a QIAGEN DNAEasy blood and tissue kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions and standard methods for DNA extraction and purification. If high-quality amplicons were not acquired, a set of ambiguous primers with universal sequencing adaptors was used (Table S2). The total volumes of PCR reactions were 10 μl (0.25 μl Promega GoTaq Flexi DNA Polymerase (5 U/μl); ddH2O; 5x Colorless buffer; 2 mM MgCl2; 0.2 mMdNTP; 0.5 μM of each primer), with 1–2 μ DNA template. PCR thermal conditions are shown in (Table S2). PCR products were purified using ExoSAP-IT (Thermo Fisher Scientific). Sequencing reactions for both strands of the amplified genes were performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). Products were sequenced using Applied Biosystems automated sequencer.

Sequences were amplified from cytochrome *c* oxidase subunit I (COI), 16S rRNA, the D3 region of 28S rRNA (28S D3 rRNA) and two segments of 18S rRNA There was no overlap of the two 18S rRNA fragments in some taxa. Therefore, the two fragments were treated as separate datasets (called 18S part1 and part2) in all analyses. Sequence contigs were assembled and visually inspected for ambiguous sites in Sequencher v. 4.5 (Gene Codes, Ann Arbor, Michigan).

QUANTIFICATION AND STATISTICAL ANALYSIS

Sequence alignments

Alignment was conducted using the MUSCLE [54] algorithm implemented in MEGA v. 6 [50] with the following settings: −400 gap opening penalty, −50 gap extension penalty. We used GBlocks V.0.91b [51] to test and where required to eliminate poorly aligned positions in the original alignments and used this dataset for an alternative analysis (https://doi.org/10.2581/zenodo.2642215).

Since saturation in substitutions can lead to incorrect phylogenetic inferences [55], the positions 1-3 were evaluated for substitution saturation by DAMBE V 5.2.13 [52] in the whole dataset. Saturation was not observed for any but the third position in only the COI dataset. As there was no conflict in topology of the separate gene trees (https://doi.org/10.2581/zenodo.2642215) we ran the analysis with all three positions included.

Phylogenetic analyses

Models of evolution for each sequence set (Table S3) were selected in MEGA v. 6 [50] based on Akaike Information Criterion. Preliminary analysis of single gene sets was unable to recover stable clades at different depths of the tree but did not show any conflict among the separate gene trees (https://doi.org/10.2581/zenodo.2642215). Therefore, phylogenetic Bayesian analyses (BA) were conducted on the concatenated dataset in MrBayes 3.2.1 [36]. Model parameter values for the partitions were estimated independently using the "unlink" command and relative site-specific rates for all gene fragments were estimated by setting the prior for "ratepr" to "variable." For all analyses, Markov Chain Monte Carlo (MCMC) sampling was conducted with two independent and

simultaneous runs for 10,000,000 generations. Trees were saved every 1000 generations. Likelihood values and effective sample size were observed with Tracer v1.4 [45], and all trees sampled before the likelihood values stabilized were discarded as burn-in. Stationarity was reassessed using the convergence diagnostics in MrBayes (i.e., the average standard deviation of split frequencies (values < 0.01) and the potential scale reduction factor (values ≈ 1.00)). A burn-in of 25% of all sampled trees was sufficient to ensure that suboptimal trees were excluded. The remaining trees were used to construct a 50% majority rule consensus tree.

Bayesian and other trees were formatted for presentation using either TreeView (Win32) 1.6.6 [46], FigTree 1.4.1 [47], or Mesquite 3.5 [48]. In order to test the robustness of our dataset we performed additional analyses using different outgroups. We found no effect on topology and support values for the ingroup clades (see https://doi.org/10.2581/zenodo.2642215 for a selection of the closest outgroup taxa). Removing *Paracimex* had no effect on the relationships of other taxa confirming the absence of long-branch attraction [21].

In order to compare the tree from Bayesian inference with Maximum Likelihood (ML) analysis we ran the same partitioned dataset by using RAxML 7.4.2 [49]. Since RaxML does not allow the use of mixed nucleotide models, we used the GTR gamma invariant (GTRGAMMAI) for all partitions. ML with rapid bootstrap was performed in 1000 iterations and obtained bootstrap values were placed on a consensus tree (https://doi.org/10.2581/zenodo.2642215).

Molecular Dating

We used BEAST 1.8.4 [31] with 82 sequences, including 20 outgroups to infer the divergence dates of the sequences under a Yule speciation process (a pure birth process) and an uncorrelated relaxed molecular clock [31].

First, we constrained the Cimicoidea as a monophyletic group and used a lognormal prior mean age of 152.2 million years (Ma) with standard deviation 0.2 Ma as calibration point for the group based on a fossil flower bug (Heteroptera: Cimicomorpha: Cimicoidea: Vetanthocoridae) from the late Jurassic [25]. In this analysis, we wanted to test if our molecular dating of the family Cimicidae is in concordance with oldest known cimicid fossil, *Quasicimex eilapinastes* Engel, 2008 from the mid Cretaceous (ca. 100 MYA) [6]. Our estimates placed the origin of Cimicidae at 93.8 MYA with a 95% highest probability density interval of 56-137 MYA (tree not shown). Accepting the fossil as a proxy for the minimum age of the Cimicidae, this clock estimate appeared as a reasonable result. To better account for variable evolutionary rates over the whole tree, we used the minimum age of *Q. eilapinastes* as an additional calibration point, setting a lognormal prior with a mean of 102.5 MYA and standard deviation 0.06 Ma for the diversification of the Cimicidae. The root in both analyses was given a weak uniform prior ranging from 0 to 350 MYA. We ran two successive MCMC chains with 100 million generations, sampling every 1000 generations. All chains had reached equilibrium at two million generations. When discarding 20% of the initial tree samples the consensus trees from each run produced the same topologies and the same branch support. We pooled samples from the two runs with the program "logcombiner" implemented in BEAST [31] by discarding 50% of the initial trees from each run and computed a consensus chronogram based on 10000 resampled trees. Parameter estimates, including posterior probabilities and mean node ages with highest probability density intervals, were calculated in TreeAnnotator (implemented in BEAST [31]) and displayed with FigTree [47].

These model settings were conservative and returned a stem age of 121.6 MYA and a crown group age of 102.2 MYA for the Cimicidae (Figure 2). Running the analysis with a birth-death instead of a Yule model, and using uniform instead of lognormal priors returned a mean estimate of 145 MYA for the stem of Cimicidae and 121 Ma for the first divergence of the family.

Our phylogeny corresponds very well with a cladistic analysis [32] placing the Vetanthocoridae within the Cimicoidea. To explore effects of earlier ideas [32] that the vetanthocorid fossil may represent the sister group of all other Cimicoidea, we defined the divergence of Cimicoidea and Nabidoidea as a calibration point, leaving the Microphysidae, Joppelicidae and Miridoidea as members of the outgroup, in accordance with our previous findings of branching patterns. Using a uniform prior with minimum age 152.2 million years for the split of Cimicoidea from Nabidoidea in MCMC runs of up to 18 million generations returned an almost identical mean age of the Cimicidae stem and of the crown group (https://doi.org/10.5281/zenodo.2642215).

Ancestral host character state reconstruction

We mapped ancestral host characters on the tree with time estimated nodes. We used Mesquite version 3.5 [48] to prune the outgroup taxa from the tree and to collapse zero-length terminal branches. We coded terminal taxa with discrete trait characters according to the known host groups of each species: bats, birds (divided into Neoaves and Galloanseres) and humans. We then used the 'trace ancestral character' function to estimate ancestral states of nodes with maximum likelihood (Figure 3A). A simple one-parameter Markov model [56], implemented in [36] was applied with these calculations, estimating the rate of state changes directly from the data [48]. In a second approach, we coded terminal taxa with the discrete trait characters 'specialist' or 'generalist' (Figure 3B). We then used the 'trace ancestral character' function to estimate ancestral states of nodes with maximum parsimony.

We also used the number of currently known host genera for each of the cimicid species as a meristic character. We traced the character states on the phylogeny using linear meristic parsimony reconstruction with Mesquite (Figure S1). In addition, we inferred ancestral states at ancestral nodes using the full hierarchical Bayesian approach (integrating uncertainty concerning topology and other model parameters) as described in [56] and integrated in MrBayes 3.2. The ancestral host character for the selected lineages (i.e., Cimicinae and (Haematosiphoninae + Cacodminae) at the KT boundary (the time of their assumed first colonization of bats) was also inferred using the full hierarchical Bayesian approach in MrBayes 3.2 [36]. All terminal taxa not belonging to one of these two lineages were coded as character "unknown host."

DATA AND SOFTWARE AVAILABILITY

Data are archived at https://doi.org/10.5281/zenodo.2642215.

The Bayesian analysis of individual gene trees (18S rDNA 2 parts, COI, 16S rDNA, 28S D3 rDNA) can be found at https://doi.org/10.5281/zenodo.2642215. The Neighbor-Joining analysis using strict and relaxed GBlock alignments of all molecular markers separately can be found at https://doi.org/10.5281/zenodo.2642215. The MrBayes consensus tree using one representative species of the closest phylogenetic outgroups can be found at https://doi.org/10.5281/zenodo.2642215. The Maximum Likelihood analysis of the combined molecular dataset can be found at https://doi.org/10.5281/zenodo.2642215. The Alignment file is available at https://doi.org/10.5281/zenodo.2642215. The Chronogramme using different a different assumptions of the taxonomic placement of the fossil is available at https://doi.org/10.5281/zenodo.2642215.

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Supplemental Information

Bedbugs Evolved before Their Bat Hosts and Did Not Co-speciate with Ancient Humans

Steffen Roth, Ondřej Balvín, Michael T. Siva-Jothy, Osvaldo Di Iorio, Petr Benda, Omar Calva, Eduardo I. Faundez, Faisal Ali Anwarali Khan, Mary McFadzen, Margie P. Lehnert, Richard Naylor, Nikolay Simov, Edward H. Morrow, Endre Willassen, and Klaus Reinhardt

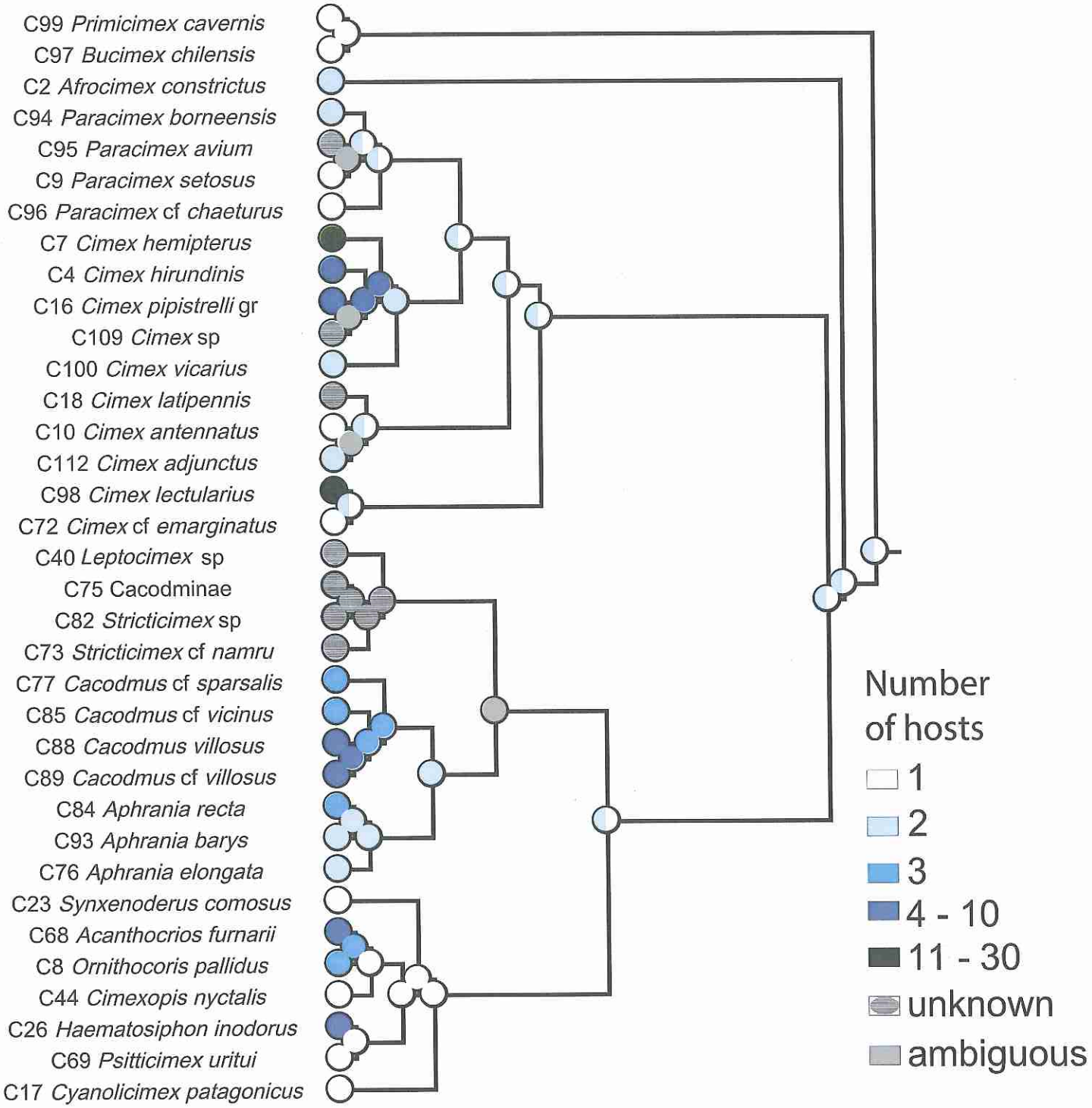
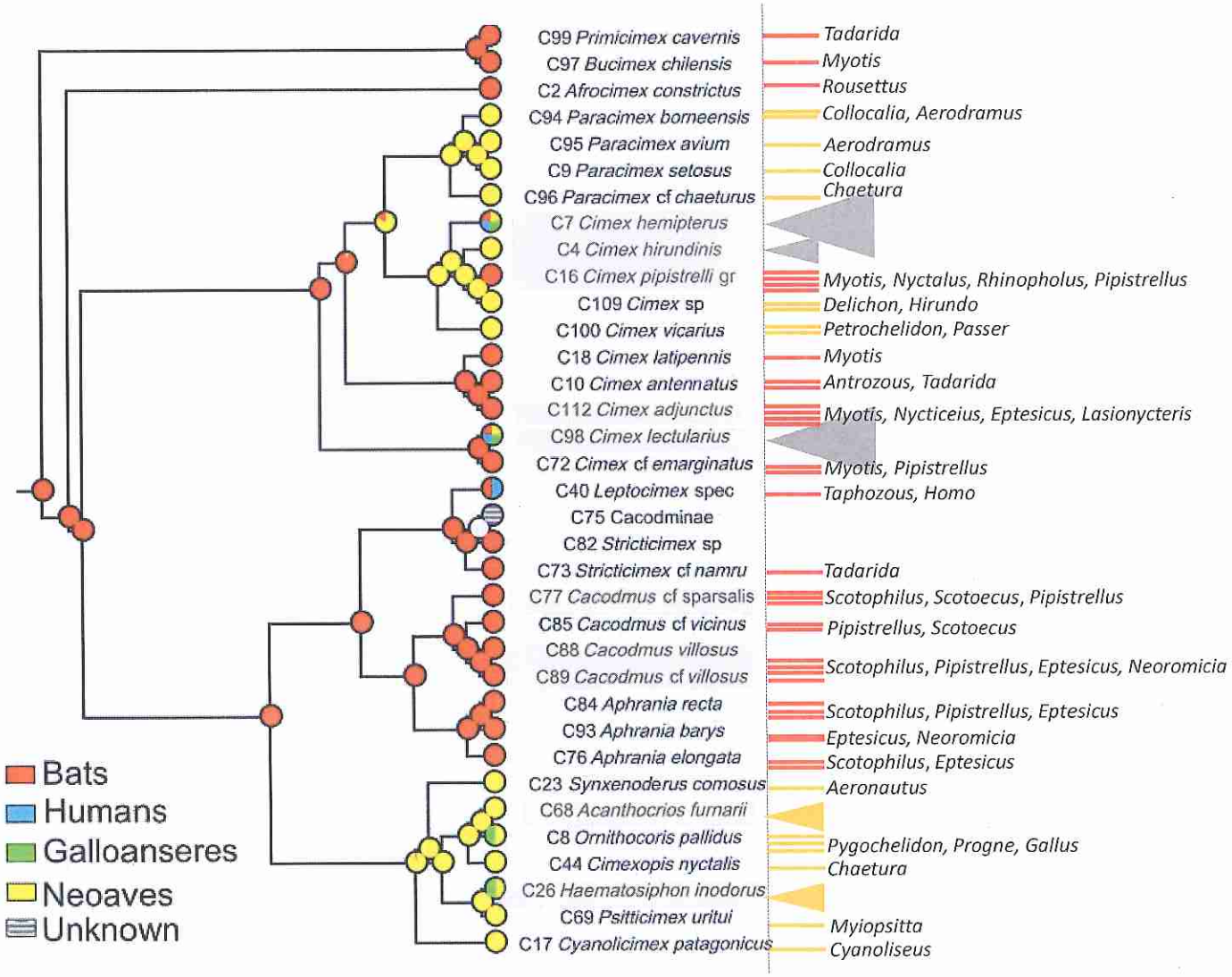

C99 Primicimex cavernis
C97 Bucimex chilensis
C2 Afrocimex constrictus
C94 Paracimex borneensis
C95 Paracimex avium
C9 Paracimex setosus
C96 Paracimex cf chaeturus
C7 Cimex hemipterus
C4 Cimex hirundinis
C16 Cimex pipistrelli gr
C109 Cimex sp
C100 Cimex vicarius
C18 Cimex latipennis
C10 Cimex antennatus
C112 Cimex adjunctus
C98 Cimex lectularius
C72 Cimex cf emarginatus
C40 Leptocimex sp
C75 Cacodminae
C82 Stricticimex sp
C73 Stricticimex cf namru
C77 Cacodmus cf sparsalis
C85 Cacodmus cf vicinus
C88 Cacodmus villosus
C89 Cacodmus cf villosus
C84 Aphrania recta
C93 Aphrania barys
C76 Aphrania elongata
C23 Synxenoderus comosus
C68 Acanthocrios furnarii
C8 Ornithocoris pallidus
C44 Cimexopis nyctalis
C26 Haematosiphon inodorus
C69 Psitticimex uritui
C17 Cyanolicimex patagonicus
Number of hosts
1
2
3
4 - 10
11 - 30
unknown
ambiguous

Figure S1. Evolution of the host spectrum in cimicids using the currently known number of host genera, Related to Figure 3, STAR Methods. The ancestral state is reconstructed as 1 or 2 host species, confirming ancestral host specialization. The methods are exactly those used for Figure 3.


C99 Primicimex cavernis
C97 Bucimex chilensis
C2 Afrocimex constrictus
C94 Paracimex borneensis
C95 Paracimex avium
C9 Paracimex setosus
C96 Paracimex cf chaeturus
C7 Cimex hemipterus
C4 Cimex hirundinis
C16 Cimex pipistrelli gr
C109 Cimex sp
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C18 Cimex latipennis
C10 Cimex antennatus
C112 Cimex adjunctus
C98 Cimex lectularius
C72 Cimex cf emarginatus
C40 Leptocimex spec
C75 Cacodminae
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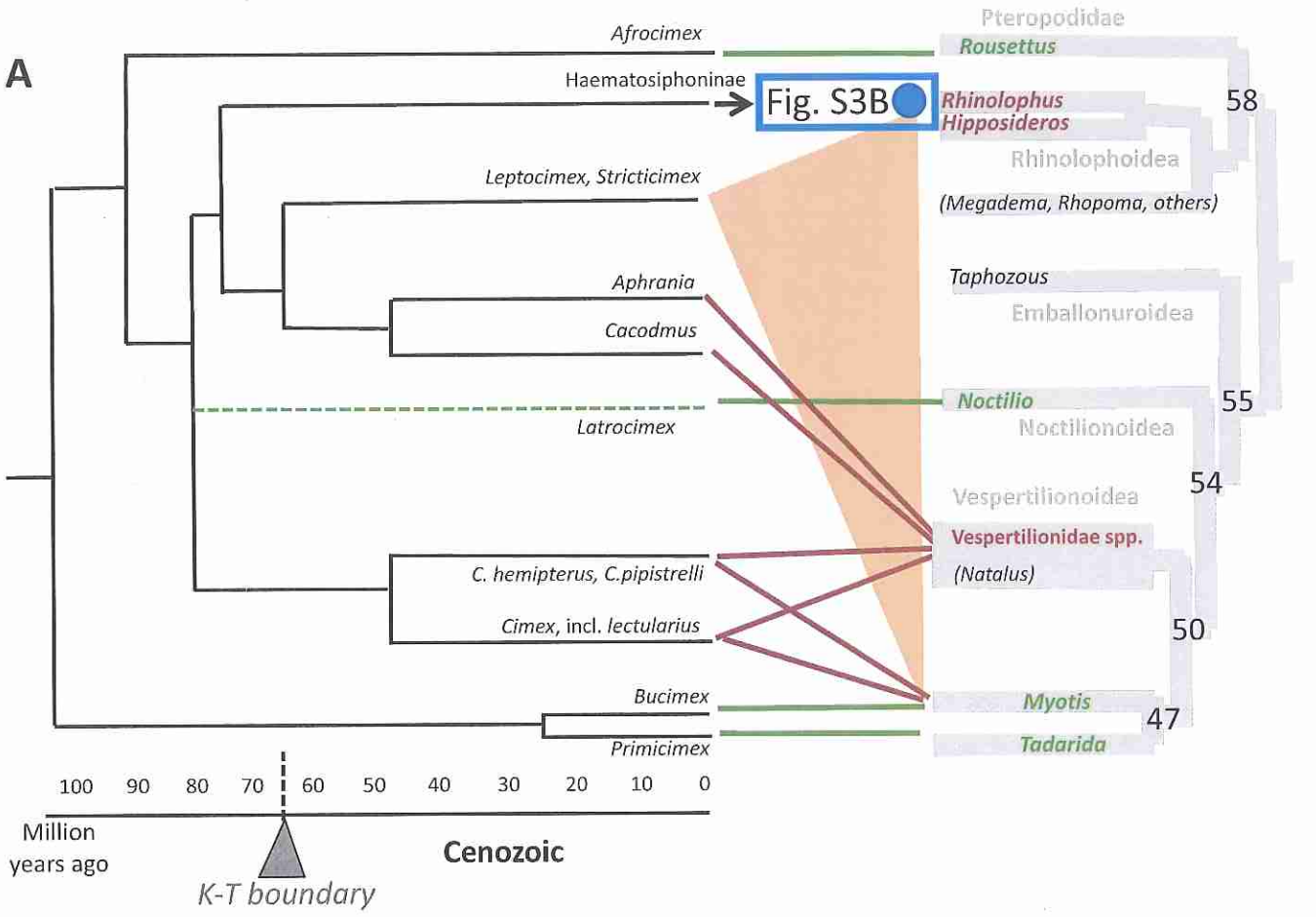
C68 Acanthocrios furnarii
C8 Ornithocoris pallidus
C44 Cimexopis nyctalis
C26 Haematosiphon inodorus
C69 Psitticimex uritui
C17 Cyanolicimex patagonicus
Tadarida
Myotis
Rousettus
Collocalia, Aerodramus
Aerodramus
Collocalia
Chaetura
Myotis, Nyctalus, Rhinopholus, Pipistrellus
Delichon, Hirundo
Petrochelidon, Passer
Myotis
Antrozous, Tadarida
Myotis, Nycticeius, Eptesicus, Lasionycteris
Myotis, Pipistrellus
Taphozous, Homo
Tadarida
Scotophilus, Scotoecus, Pipistrellus
Pipistrellus, Scotoecus
Scotophilus, Pipistrellus, Eptesicus, Neoromicia
Scotophilus, Pipistrellus, Eptesicus
Eptesicus, Neoromicia
Scotophilus, Eptesicus
Aeronautus
Pygochelidon, Progne, Gallus
Chaetura
Myiopsitta
Cyanoliseus
Bats
Humans
Galloanseres
Neoaves
Unknown

Figure S2. Host reconstruction using a stricter definition of generalism, Related to Figure 3. Host generalism is defined as utilizing more than three host genera (species shaded in grey) and plotted against a cimicid tree using more relaxed definition of generalism (Figure 3). The number of lines next to the bug species name indicates the number of currently known host genera. Triangles indicate more than four genera, the size of the triangle indicating the number of host genera. The host genera are listed. The host spectrum was obtained from the same sources as for Figure 3, with an additional record for *C. sparsilis* on domestic dog [S1].


A
Afrocimex
Haematosiphoninae
Fig. S3B
Leptocimex, Stricticimex
Aphrania
Cacodmus
Latrocimex
C. hemipterus, C.pipistrelli
Cimex, incl. lectularius
Bucimex
Primicimex
Pteropodidae
Rousettus
Rhinolophus
Hipposideros
Rhinolophoidea
(Megadema, Rhopoma, others)
Taphozous
Emballonuroidea
Noctilio
Noctilionoidea
Vespertilionoidea
Vespertilionidae spp.
(Natalus)
Myotis
Tadarida
58
55
54
50
47
100 90 80 70 60 50 40 30 20 10 0
Million years ago
K-T boundary
Cenozoic

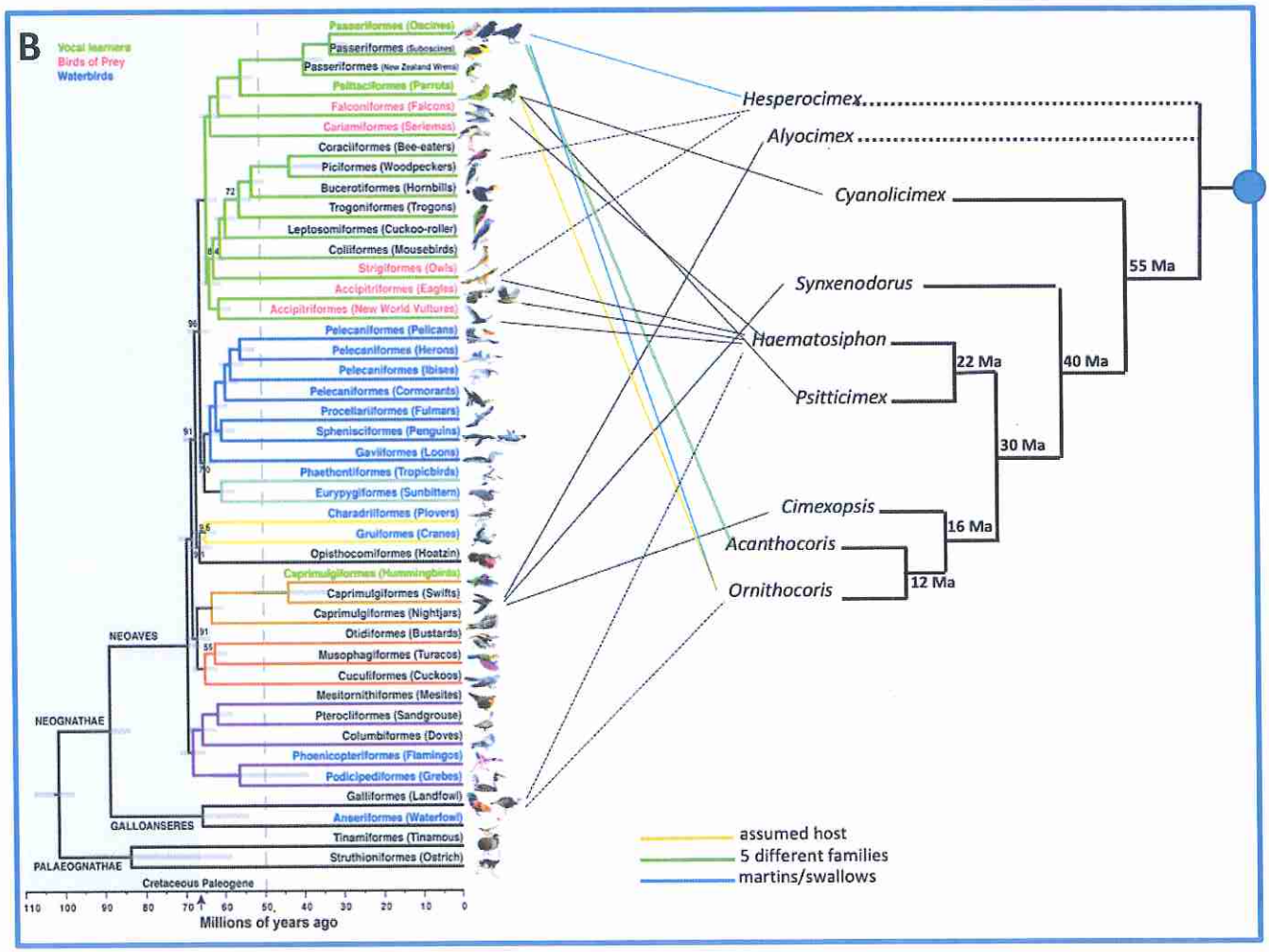

B
Vocal learners
Birds of Prey
Waterbirds
Passeriformes (Oscines)
Passeriformes (Suboscines)
Passeriformes (New Zealand Wrens)
Psittaciformes (Parrots)
Falconiformes (Falcons)
Cariamiformes (Seriemas)
Coraciiformes (Bee-eaters)
Piciformes (Woodpeckers)
Bucerotiformes (Hornbills)
Trogoniformes (Trogons)
Leptosomiformes (Cuckoo-roller)
Coliiformes (Mousebirds)
Strigiformes (Owls)
Accipitriformes (Eagles)
Accipitriformes (New World Vultures)
Pelecaniformes (Pelicans)
Pelecaniformes (Herons)
Pelecaniformes (Ibises)
Pelecaniformes (Cormorants)
Procellariiformes (Fulmars)
Sphenisciformes (Penguins)
Gaviiformes (Loons)
Phaethontiformes (Tropicbirds)
Eurypygiformes (Sunbittern)
Charadriiformes (Plovers)
Gruiformes (Cranes)
Opisthocomiformes (Hoatzin)
Caprimulgiformes (Hummingbirds)
Caprimulgiformes (Swifts)
Caprimulgiformes (Nightjars)
Otidiformes (Bustards)
Musophagiformes (Turacos)
Cuculiformes (Cuckoos)
Mesitornithiformes (Mesites)
Pteroclidiformes (Sandgrouse)
Columbiformes (Doves)
Phoenicopteriformes (Flamingos)
Podicipediformes (Grebes)
Gaviiformes (Landfowl)
Anseriformes (Waterfowl)
Tinamiformes (Tinamous)
Struthioniformes (Ostrich)
NEOAVES
NEOGNATHAE
GALLOANSERES
PALAEOGNATHAE
Cretaceous Paleogene
110 100 90 80 70 60 50 40 30 20 10 0
Millions of years ago
Hesperocimex
Alyocimex
Cyanolicimex
Synxenodorus
Haematosiphon
Psitticimex
Cimexopsis
Acanthocoris
Ornithocoris
55 Ma
40 Ma
30 Ma
22 Ma
16 Ma
12 Ma
assumed host
5 different families
martins/swallows

Figure S3. Host relationships (tanglegram) of the Cimicidae, Related to Figure 3. A. Cimicidae parasitic on bats. Specialists, defined as using only one species or genus as hosts, are connected by green lines to their hosts, generalists with a wider range of host taxa are shown with red connecting lines: *Leptocimex* and *Stricticimex* utilize hosts (except *Noctilio*) that phylogenetically are wide apart (orange). Bat phylogeny according to [S2], host spectrum after [S3–S6, Table S1]. **B. Haematosiphoninae.** Primary hosts (solid line) and secondary hosts (long dashed line) [after S7]. Dotted branches are species that were not analyzed in our study. The Haematosiphoninae, originating at blue dot, diverged around 50 MYA. As other bird-parasitic lineages, *Paracimex* (diverging around 15 MYA) or *Cimex vicarius* (diverging around 18 MYA), they appeared long after their respective swift or swallow host groups arose in the early Eocene [S8, S9]. Phylogram of birds from [S10]. Hosts were compiled from [S3–S6, Table S1].

ID/Species	Country, Locality	Host	Date	Legit/Coll	stage	Genbank accession no.				
						COI	16S	18S part 1	18S part 2	28S
Primicinae: *Primicimex cavernis* Barber, 1941										
C41	USA, Texas, Vel Verde County, Fern cave	*Tadarida brasiliensis*	27.06.2003	Jim Kennedy	n	MG596838	MG596875	n.a.	n.a.	n.a.
C99	Mexico, Sonora Dessert	*Tadarida brasiliensis*	Sept. 2015	Omar Calva	n	MG596839	MG596876	MG978398	MG978398	MG763734
Primicinae: *Bucimex chilensis* Usinger 1963										
C97	Chile, R. de los Rios Mariquina, Valdivia, Pelchuquin	unknown	02.01.2013	J.F. Campodonico	f	MG596840	MG596877	MG978399	MG978399	MG763735
Afrocimicinae: *Afrocimex constrictus* Ferris and Usinger 1957										
C1	Kenya, Mount Elgon National Park	*Rousettus aegyptiacus*	March 2005	Reinhardt et al. 2007	?	MG596804	MG596841	MG978357	MG978357	MG763685
C2	Kenya, Mount Elgon National Park	*Rousettus aegyptiacus*	March 2005	Reinhardt et al. 2007	?	MG596805	MG596842	MG978358	MG978358	MG763686
C3	Kenya, Mount Elgon National Park	*Rousettus aegyptiacus*	March 2005	Reinhardt et al. 2007	?	MG596806	MG596843	MG978359	MG978359	MG763687
Haematosiphoninae: *Ornithocoris pallidus* Usinger 1959										
C8	USA, South Carolina, Ilse of Palms			Mary Pringle	n	MG596827	MG596863	MG978382	MG978382	MG763715
C45	USA, South Carolina, Ilse of Palms	Martin (nest)	August 2010	Mary Pringle	n	MG596828	MG596864	MG978383	MG978383	MG763716
Haematosiphoninae: *Haematosiphon inodorus* (Dugés 1892)										
C26	USA, Idaho, Snake River Birds of Prey National Conservation Area	*Falco mexicanus* (nest)	1994	Mary Mc Fadzen	n	MG596829	MG596865	MG978384	MG978384	MG763717
Haematosiphoninae: *Acanthocrios furnarii (Cordero and Vogelsang 1928)*										
C68	Brazil, Canuelas	*Furnarius rufus* (nest)	12.10.2010	Osvaldo Di Iorio	m	MG596830	MG596866	MG978385	MG978385	MG763718
Haematosiphoninae: *Psitticimex uritai* (Lent and Abalos 1946)										
C69	Argentina, Buenes Aires, Junin	*Myiopsitta monachus*	20.06.2008	Osvaldo Di Iorio	n	MG596831	MG596867	MG978386	MG978386	MG763719
Haematosiphoninae: *Synxenoderus camosus* List 1925										
C23	USA, California, Los Angeles County	*Aeronautes saxatalis* (nest)	02.06. 2000	C.T. Collins	f	MG596832	MG596868	MG978387	MG978387	MG763720
Haematosiphoninae: *Cyanolicimex patagonicus* Carpintero, Di Iorio, Masello and Turienzo 2010										
C17	Argentina, Rio Negro, El Condor (Patagonia)	*Cyanoliseus patagonus*	20.12.2003	Petra Quillfeldt	n	MG596833	MG596869	MG978388	MG978388	MG763721
Haematosiphoninae: *Cimexopis nyctalis* List 1925										
C12, C44	USA, Texas, Travis County, NW of Austin, TX.	*Chaetura pelagica*	Sept. 1997	Paul D. Kyle	n	n.a.	MG596870	MG978389	n.a.	MG763722
Cacodminae: *Leptocimex duplicatus* Usinger 1959										
C40	Israel, Kafia Cave, 31°44'N 35°28' E		12.10.2012	Shmuulik Landau	n	MG596810	MG596847	MG978365	MG978365	MG763694
Cacodminae: *Stricticimex* cf. *namru* Usinger 1960										
C73	Iran, Ilam Province, Dehloran Cave (nymph)	mixed colony of *Asellia tridens*, *Rhinopoma microphyllum*, and *R. hardwickii*	17.10.2011	Petr Benda	n	MG596811	MG596848	MG978366	MG978366	MG763695
Cacodminae: *Stricticimex* spec.										
C82	Oman, Al Batinah Ash Shamal Province, Ghab, Wadi Al Hawasina (nymph)	*Nyctinomus thomasi*	07.04.2011	Petr Benda	n	MG596817	MG596853	MG978372	MG978372	MG763702
Cacodminae: Cacodminae spec.										
C75	Oman, Ash Sharqiyah Al Janub Province, Jaalan Bani Bu Ali (nymph)	*Taphozous nudiventris*	02.04.2011	Petr Benda	f	MG596814	n.a.	MG978368	MG978368	MG763697
Cacodminae: *Cacodmus* cf. *sparsilis* (Rothschild 1912)										
C77	Oman, Dhofar Province, Wadi Hannah	*Pipistrellus dhofarensis*	13.10.2008	Petr Benda	f	MG596813	MG596850	MG978369	MG978369	MG763699
Cacodminae: *Cacodmus vicinus* Horvath1934										
C103	Jordan, Zarqa Province, Azraq Wetland Reserve	*Pipistrellus kuhlii*	13.10.2008	Petr Benda	?	KF018762	KF018728	KF018714	KF018714	MG763701
C49	Spain, Caceres province, Plasencia	*Pipistrellus* sp.	19.09.2006	unknown, coll. Margaret Lehnert	m	MG596816	MG596852	MG978371	MG978371	MG763700
C85	Senegal, Fatick Province, Farbala Reserve	*Scotoecus hirundo*	14.02.2012	Radek Lučan	f	MG596819	MG596855	MG978374	MG978374	MG763705
Cacodminae: *Cacodmus villosus* (Stål 1855)										
C5	Kenya, Mount Elgon National Park	unknown	20.03.2005	Michael Siva-Jothy	m	MG596815	MG596851	MG978370	MG978370	MH181391
C87	Ethiopia, 4 km S of Korem	*Pipistrellus hesperidus*	28.10.2012	Petr Benda	?	MG596821	MG596857	MG978376	MG978376	MG763707
C88	Ethiopia, Suha Menugelas Forest	*Pipistrellus hesperidus*	25.10.2014	Petr Benda	m	MG596822	MG596858	MG978377	MG978377	MG763708
C89	Namibia, Kavango, East Province, Ncaute	*Neoromicia capensis*	22.01.2012	Petr Benda	f	MG596823	MG596859	MG978378	MG978378	MG763709

C90	Namibia Oshikoto Province, Ghaub farm	*Neoromicia capensis*	15.07.2014	Petr Benda	f	MG596824	MG596860	MG978379	MG978379	MG763710
Cacodminae: *Aphrania burys* Jordan and Rothschild 1912										
C86	Namibia, Zambezi Province, Kongola	*Neoromicia capensis*	28.01.2012	Petr Benda	?	MG596820	MG596856	MG978375	MG978375	MG763706
C93	South Africa, Northern Cape Province, Tswalu Private Nature Reserve	*Neoromicia capensis*	01.01.2009	Samantha Stoffberg	f	MG596825	MG596861	MG978380	MG978380	MG763711
Cacodminae: *Aphrania recta* Ferris and Usinger1957										
C78	Mauritania, Brakna Province, Boguć	*Nycticeinops schlieffenii*	14.10.2010	Petr Benda	?	KF018764	KF018730	KF018716	KF018716	MG763704
C84	Senegal, Fatick Province, Fathala Reserve	*Neoromicia* cf. *guineensis*	14.02.2012	Radek Lučan	f	MG596818	MG596854	MG978373	MG978373	MG763703
Cacodminae: *Aphrania elongata* Usinger 1966										
C74	Senegal, Fatick Province, Fathala Reserve	unknown	February 2012	Radek Lučan	?	MG596812	MG596849	MG978367	MG978367	MG763696
C76	Mauritania, Brakna Province, Boguć	*Scotophilus leucogaster*	14.10. 2010	Petr Benda	?	KF018763	KF018729	KF018715	KF018715	MG763698
Cimicinae: *Cimex pipistrelli* group: Europe										
C30	Laboratory population (5 yrs), originally from Hanau (Germany)	*Nyctalus noctula*	05.06.2004	Klaus Reinhardt, Dieter Koch	f	MG596834	MG596871	MG978390	MG978390	MG763723
C106	U.K. Rindleford, Bridgnorth, Shropshire	*Pipistrellus* spec.	14.xii.1999	John Mason	?	GU985534	GU985556	MG978393	MG978393	MG763726
C16	Huelva, Spain	bat	1 July 2003	Juan Quetglas	?	MG596835	MG596872	MG978392	MG978392	MG763725
C108	Bulgaria, Červen, Ruse region	*Nyctalus noctula*	1.10.2005	Ivailo Borissov	?	GU985530	GU98555	MG978391	MG978391	MG763724
Cimicinae: *Cimex* sp. *cf Cimex pipistrelli* group (see ref (*54*) for details)										
C109	Japan, Konda, Sasayama-City, Hyogo prefecture	*Delichon dasypus*, *Hirundo daurica japonica*	9.3.2008	Nobuhiko Kanaoka	?	GU985542	GU985564	KF018708	KF018708	MG763727
Cimicinae: *Cimex adjunctus* Barber 1939										
C110	USA, Washington county, North Carolina1	*Nycticeius humeralis*	7.6.2005	Matina Kalcounis-Ruppell	?	GU985536	GU985558	KF018712	KF018712	MG763737
C111	USA, Galesburg, Kalamazoo county, Michigan	*Eptesicus fuscus*	6.7.2005	Lee Johnson	?	GU985535	GU985557	MG978400	MG978400	MG763736
C112	USA, Fulton, Kalamazoo county, Michigan	*Eptesicus fuscus*	19.6.2005	Lee Johnson	?	GU985537	GU985559	MG978401	MG978401	MG763738
Cimicinae: *Cimex* cf. *antennatus* Usinger and Ueshima 1965										
C10	USA, Antelope Valley, California		15.8.2002	A.C. Lohmann		KF018760	KF018732	KF018718	KF018718	MG763739
Cimicinae: *Cimex latipennis* Usinger and Ueshima 1965										
C18	Canada, Hope, British Columbia, Canada	*Myotis lucifugus*	unknown	T. Luszczell	?	KF018758	KF018734	KF018720	KF018720	MG763740
C19	Canada, Hope, British Columbia, Canada	*Myotis volans*	unknown	T. Luszczell	?	KF018757	KF018733	KF018719	KF018719	MG763741
Cimicinae: *Cimex hemipterus* Fabricius 1803										
C7	Kenya, Mombasa	Human		Oliver Otti	m	MG596826	MG596862	MG978381	MG978381	MG763712
C104	Malaysia, Melaca	human	15.12.2011	Hana Šípková	?	KF018754	KF018724	KF018710	KF018710	MG763713
C105	India, Tamil Nadu	human	6.12.2010	Robert Vlk	?	KF018755	KF018725	KF018710	KF018710	MG763714
Cimicinae: *Cimex lectularius* (Linnaeus 1758)										
C113	Czech Republic, Olomouc, stock	human	autumn 2005	Libor Mazánek	?	GU985524	GU985546	KF018711	KF018711	MG763730
C107	Czech Republic, Brandýs nad Orlicí	*Myotis myotis*	21.6.2006	Ondřej Balvín		GU985526	GU985548	KF018711	KF018711	MG763729
C24	long standing (>40 yrs) laboratory stock, London, UK	human		Klaus Reinhardt	f	MG596836	MG596873	MG978394	MG978394	MG763728
C98	Iran, Golestan province, picnic grass field	unknown host	27.05.2006	Jiří Hájek, Pavel Chvojka	?	MF680527	MF680518	MG978395	MG978395	MG763731
Cimicinae: *Cimex* cf. *emarginatus* Simov, Ivanova & Schunger, 2006										
C72	Bulgaria, Malashevska planina Mt., Monastery of the Saints Archangels, N 41.8564/ E22.99197	*Myotis* cf. *alcathoe*	10.09.2011	Leg. B. Petrov, I. Alexandrova, coll Nikolay Simov	f	MG596837	MG596874	MG978396	MG978396	MG763732
C80	Morocco, Bouhachen, Rif	*Pipistrellus pipistrellus*	06.05.2013	Tomáš Bartonička	m	MF680526	MF680517	MG978397	MG978397	MG763733
Cimicinae: Cimex (former *Oeciacus) hirundinis* (Lamarck 1816)										
C4	Switzerland	unknown	unknown	Pierre Bize	n	MG596808	MG596845	MG978363	MG978363	MG763692
C46	Czech Republic, Žitec, Jindřichův Hradec	*Delichon urbica*	23.11.2007	Jaroslav Cepák		MG596809	MG596846	MG978364	MG978364	MG763693
Cimicinae: Cimex (former *Oeciacus) vicarius* (Horvath 1890)										
C100	USA, Sarben, Keith County, Nebraska	*Petrochelidon pyrrhonota*	2.10.2008	Charles R. Brown	n	GU985541	GU985563	KF018709	KF018709	MG763742
C101	USA, Keystone, Keith County, Nebraska	*Petrochelidon pyrrhonota*	3.10.2008	Charles R. Brown	n	KF018753	KF018723	KF018709	KF018709	MG763743
C102	USA, Keystone, Keith County, Nebraska	*Petrochelidon pyrrhonota*	3.10.2008	Charles R. Brown	n	KF018752	KF018722	KF018709	KF018709	MG763744
Cimicinae: *Paracimex setosus* Ferris and Usinger 1957										
C9	Malaysia	*Aerodramus vanikorensis* or *fuciphagus*		Dale Clayton	n	KF018761	KF018735	KF018721	KF018721	MG763689
Cimicinae: *Paracimex borneoensis* Usinger 1959										

C94	Malaysia, Borneo, Niah cave	*Aerodramus salangamus*	26.6.2015	Steffen Roth, Adrian Scheidt	n	KP018761	MF680519	MG978361	MG978361	MG763690
Cimicinae: *Paracimex arium* Kiritshenko 1913										
C95	Indonesia, Sumatra, Aceh, Takengon, Buffalo Cave (brass Kerto), N 4.605577/Ep6.882463, 4.5km ESE of Takengon south bank of the lake	*Aerodramus salangamus*	3.6.2105	Adrian Scheidt	n	MG596807	MG596844	MG978360	MG978360	MG763688
Cimicinae: *Paracimex cf elmeturus* Ueshima 1968										
C96	China, Cave near Jiangshui, Yunnan province	*Aerodramus brevirostris*	May 2015	Ondřej Balvín	n	MF680531	MF680520	MG978362	MG978362	MG763691
OUTGROUP TAXA - DNA sequences from GenBank						**COI**	**16S**	**18SI**	**18SII**	**28S**
OGA1	Anthocoridae: *Blaptostethus aurivillus* Kazutaka 2008				?	KF36463	GQ258388	GQ258400	GQ258400	GQ258440
OGA2	Anthocoridae: *Scoloposcelis albodecussata* Yamada, Kazutaka & Hirowatari, Toshiya 2005				?	GQ292129	GQ258376	GQ258422	GQ258422	GQ258457
OGA3	Anthocoridae: *Xylocoris cerealis* Yamada & Yasunaga 2006				?	GQ292172	GQ258384	GQ258395	GQ258395	GQ258459
OGA4	Anthocoridae: *Buchananiella crassicornis* Carayon 1958				?	GQ292145	GQ258364	GQ258407	GQ258407	GQ258441
OGA5	Anthocoridae: *Orius minutus* (Linnaeus 1758)				?	KR040183	GQ258372	GQ258417	GQ258417	GQ258452
OGA6	Anthocoridae: *Dysepicritus rufescens* (Costa 1847)				?	GQ292210	GQ258386	GQ258399	GQ258399	GQ258444
OGA7	Anthocoridae: *Amphiareus obscuriceps* (Poppius 1909)				?	GQ292178	GQ258358	GQ258393	GQ258393	GQ258429
OGA8	Anthocoridae: *Anthocoris confusus* Reuter 1884				?	KM022525	GQ258359	GQ258401	GQ258401	GQ258431
OGNab1	Nabidae: *Prostemma div.* spp.				?	JQ782833	JQ782833	JQ782787	JQ782787	JQ7828081
OGNab2	Nabidae: *Himacerus apterus* (Fabricius 1798)				?	KR034788	GQ258381	GQ258425	GQ258425	GQ258435
OGNab3	Nabidae: *Nabis flavomarginatus* Scholtz 1847				?	KM022694	GQ258380	GQ258424	GQ258424	GQ258433
OGNab4	Nabidae: *Nabis stenoferus* Hsiao, 1964				?	GQ292211	GQ258379	GQ258426	GQ258426	GQ258434
OG	Joppeicidae: *Joppeicus paradoxus* Reuter, 1910				?	AY252951	AY252688	EU6831471	n.a.	AY252455
OG	Lasiochilidae: *Lasiochilus japonicus* Hiura, 1967				?	GQ292187	GQ258367	GQ258410	GQ258410	GQ258445
OG	Lyctocoridae: *Lyctocoris beneficus* (Hiura, 1959)				?	GQ292284	GQ258369	GQ258412	GQ258412	GQ258447
OG	Microphysidae: *Loricula elegantula* (Baerensprung, 1858)				?	KM022867	EU683098	EU683151	EU683151	AY252557
OG	Plokiophilidae: *Lipokophila eberhardi* Schuh, 1993				?	n.a.	AY252661	AY252148	n.a.	AY252432
OG	Curalidae: *Curalium cronini* Schuh, Weirauch, Henry & Halbert, 2008				?	n.a.	n.a.	EU683128	EU683128	n.a.
OG	Tingidae: *Eteaneus angulatus* Drake & Maa 1953				?	EF523481	EF487290	EF487311	EF487311	EF487321
OG	Miridae: *Capsus ater* (Linnaeus, 1758)				?	AY252977	AY252712	EU683117	EU683117	AY252483

Table S1. Overview of the samples obtained, their localities and corresponding Genbank admission numbers, Related to Figures 1 and 3 and STAR Methods. The samples of 34 species cover 30% of extant species described to date from 6 out of 7 recognized subfamilies, or 17 out of 26 genera described to date [S11], their localities and Genbank admission numbers. In addition to the collectors mentioned we thank M.I.M. Azhar, P. Bize, H. Brailovsky, P. Christe, A. Gueorguieva, G. Kyle, C. Lausen, R. Mally, E. McArthur, L. Mollis, J. Rasgon, W. Reeves, M. Ševčík, S. Stoffberg, and M. Webb for providing specimens or help in obtaining them. Stage m, f and n denotes male, female and nymph, respectively.

Gene	Abbrev-iation	Direction of primer	Primer name	Sequence from 5′ to 3′	Reference	Annealing temperature
Cytochrome oxidase subunit I	COI	F	Lep1Fdeg	ATTCAACCAATCATAAAGATA TNGG	[S12] modified	42°C
	COI	F	Lep1F	ATTCAACCAATCATAAAGATA TTGG	[S12]	48°C
	COI	R	Lep3R	TATACTTCAGGGTGTCCGAAA AATCA	[S12] modified	42°/48°C
	COI	F	jgHCO	TITCIACIAAYCAYAARGAYATT GG	[S13]	42°C
	COI	R	jgLCO	TAIACYTCIGGRTGICCRAARAA YCA	[S13]	42°C
16S ribosomal	16S	F	16S LR-J	TTA CGC TGT TAT CCC TAA	[S14]	48°C
	16S	R	16S LR-N	CGC CTG TTT ATC AAA AAC AT	[S15]	48°C
	16S	F	16Ar	CGCCTGTTTATCAAAAACAT	[S16]	48°C
	16S	R	16Br	CGGTCTGAACTCAGATCACG	[S16]	48°C
18S ribosomal	18S	F	18S-1	CTG GTT GAT CCT GCC AGT AGT	[S17]	48°C
	18S	R	18S-3	GGT TAG AAC TAG GGC GGT ATC T	[S17]	48°C
	18S	F	18S-2	AGA TAC CGC CCT AGT TCT AAC	[S17]	48°C
	18S	R	18S-4	GAT CCT TCT GCA GGT TCA CC	[S17]	48°C
	18S	F	329	TAATGATCCTTCCGCAGGTT	[S18]	44°/48°C
	18S	R	328	CCTGGTTGATCCTGCCAG	[S18]	44°/48°C
28s ribosomal	28S (D3)	F	1274	GACCCGTCTTGAAACACGGA	[S19]	48°C
28s ribosomal	28S (D3)	R	1275	TCGGAAGGAACCAGCTACTA	[S19]	48°C

Table S2. List of primers used and PCR conditions. Related to STAR Methods.

Gene	Sequence length (bp)	Number of missing taxa	Alignment position	Parsimony informative	Variable sites	Evolution model
COI	591-659	2	659	335	359	GTR+G+I
16S rDNA	361-519	1	571	311	395	TN93+G+I
28S rDNA	301-337	1	363	82	121	K2+H
18S rDNA part 1	561-988	2	1121	266	415	K2+G+I
18S rDNA part 2	598-697	3	711	78	144	K2+G+I

Table S3. Characteristics of sequences used. Related to STAR Methods. To implement Kimura's two-parameter model (K2) in BEAST 1.8.4 [S20] we selected the Hasegawa-Kishino-Yano (HKY) model and set "base frequencies" to "All Equal". For many taxa sampled, the two 18S fragments did not overlap. Therefore, the two fragments were analyzed separately.

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Research Article

Xenointoxication of a Rabbit for the Control of the Common Bed Bug *Cimex lectularius* L. Using Ivermectin

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Human bed bug infestations have undergone a recent global resurgence. The human antiparasitic drug ivermectin has been proposed as a strategy to help control bed bug infestations, but *in vivo* data are lacking. We allowed separate populations of the common bed bug, *Cimex lectularius* L., to feed once on a rabbit before and after it was injected subcutaneously with 0.3 mg/kg of ivermectin, and bed bug morbidity and mortality were recorded. Ivermectin levels in the rabbit were measured using high-performance liquid chromatography and mass spectroscopy. Ivermectin blood levels of ~2 ng/mL caused reductions in bed bug fecundity, and levels of >8 ng/mL caused bed bug death and long-term morbidity including reductions in refeeding, mobility, reproduction, and molting. Gut bacterial cultures from the fed bed bugs showed that ivermectin altered the bed bug gut microbiome.

1. Introduction

The common bed bug, *Cimex lectularius* Linnaeus (1758) is a cosmopolitan anthropophilic hematophagous temporary human ectoparasite [1, 2]. *Cimex lectularius* has made a recent global resurgence, and current bed bug control is significantly hampered by an increasing resistance of the insect to pesticides used in their control [3–6]. Bed bugs are arguably now one of the most important human ectoparasites in western industrialized nations, and new approaches are needed for managing infestations.

The pharmaceutical drug ivermectin is one of the most common antiparasitic drugs given to human, and is used to treat many human ectoparasites including *Pediculus humanus capitis* (head lice), *Pediculus humanus humanus* (body lice), *Pthirus pubis* (pubic lice), and *Sarcoptes scabiei* (scabies). Ivermectin is not currently used in the control of *C. lectularius*, but ivermectin has been shown to kill bed bugs at plasma levels that have been reported in humans taking the drug [7–11]. Ivermectin binds to the invertebrate glutamate-gated chloride channel causing cellular hyperpolarization leading to paralysis and death [12].

The existing research involving ivermectin and bed bugs has focused on *in vitro* feedings where blood samples are spiked with ivermectin and fed to bed bugs in an artificial feeding system [9, 10]. These *in vitro* conditions likely do not replicate *in vivo* conditions principally for two reasons: pharmacokinetic studies of ivermectin in humans report ivermectin levels as plasma concentration, but bed bugs consume whole blood meals, and the secondary metabolites of ivermectin likely have antiparasitic effects that may persist beyond the parent compound. Ivermectin added to defibrinated *in vitro* blood samples will not undergo appreciable metabolism, so any secondary metabolites with toxic effects against bed bugs cannot be studied. Any ivermectin that accumulates intracellularly in hematogenous cells under

in vivo conditions would not be measured in the plasma concentration. The *in vivo* concentration of ivermectin in the blood that is required to cause bed bug toxicity is unknown.

The objective of our study was to record bed bug mortality and the insects interest and ability to refeed, fecundity, activity level, and molting after the insects fed on a rabbit injected with ivermectin. Our secondary objectives were to correlate blood ivermectin levels in the rabbit with bed bug morbidity and mortality and to determine if ivermectin affects the bed bug gut microbiome.

2. Materials and Methods

2.1. Rabbit. We received Institutional Animal Care and Use Committee's (IACUC) approval. We used a two-year-old New Zealand white neutered male rabbit weighing 4.54 kg. Ivermectin (1% sterile solution) (Noromectin®, Norbrook Laboratories, UK) at 0.3 mg/kg was injected subcutaneously into the left shoulder of the rabbit by a veterinarian. The rabbit was trained to sit in the lap of a handler while the insects fed on a shaved left hip. The same rabbit was used for both experiments which were separated by more than a month.

2.2. Insects. Ridge-strain *C. lectularius* L. was used in the experiments. These insects were first collected in a New Haven, Connecticut, apartment in 2009 and have been maintained under laboratory conditions by feeding on a human volunteer. Populations were kept in small glass canning jars with open mouths covered by sheer fabric secured by a metal band. Inside each jar were vertical cardboard wafers where insects established natural refuges. Insects were exposed to natural daylight cycles at a temperature of ~24°C (75°F) and 40–50% RH conditions.

Featherweight surface forceps were used to move insects. Twelve populations of five males, five females, and 10 mixed age instar nymphs were placed into 55 × 25 mm (4 dram) glass vials which contained a single piece of 10 × 40 mm card for perching. To keep stress low, vials were populated with 20 fully sated insects seven days before each test and left undisturbed. A 60 × 60 mm square of sheer fabric was secured over the vial mouth with an elastic band. When inverted, the card slid to the vial mouth, allowing insects to feed through the fabric.

2.3. Feeding Experiment. Populations of 5 females, 5 males, and 10 nymphs fed once on the rabbit at specific post-injection time intervals 0 (control—just before the rabbit was given ivermectin), 1, 6, 12, 24, 36, 48, 72, 96, 120, 144, and 168 hours were taken. All bed bugs fed to repletion. The experiment was repeated twice (Test 1 and Test 2).

Test 1 recorded the health of the bed bugs up to day 45 after feeding. Test 2 recorded the health of the bed bug up to day 64 after feeding but also included a breath test on day 14—performed by gently exhaling onto the insects and observing levels of stimulation and opportunities to feed on an investigator at days 16 and 31. Bed bugs were categorized as healthy (alert and active); reactive (cognizant to stimuli without response); immobile (not cognizant and lying on back with partial limb paralysis); paralyzed (not cognizant, on back with slight muscle twitch, and slow gut pulse); and dead (no movement).

2.4. High-Performance Liquid Chromatography and Mass Spectroscopy. For Test 2, 0.5 mL of blood was drawn from the marginal ear vein at 0, 6, 24, 36, 48, and 72 hours post-ivermectin injection. This blood was centrifuged at low speed, and the whole blood sample was frozen to −52°C and shipped on dry ice to Case Western Reserve University Proteomics Core facility, Cleveland, Ohio, for testing. The ivermectin concentration was analyzed using a modified method reported previously [13]. In brief, each sample was thawed and 50 microliters of supernatant was mixed with 500 microliters of 3 : 1 v/v methanol : acetonitrile, vortexed for 20 seconds, and incubated at 4°C for 30 minutes. The solution was centrifuged at 16,000 *g* for 20 minutes, and 500 microliters of supernatant was transferred to a clean tube and speed vacuumed to dryness. It was then reconstituted with 50 microliters of reconstitute solvent (0.5 mM ammonium formate; 0.1% formic acid in 50% methanol) and centrifuged at 16,000 *g* for 20 minutes. Five microliters were used for high-performance liquid chromatography (HPLC) and mass spectrometry (MS). The chromatography was performed with a reversed-phase C18 column (Atlantis dC18 column, 50 × 2.1 mm, 3 μm, Waters). Ivermectin was separated from blood endogenous components using 10% 0.5 mM ammonium formate containing 0.1% formic acid in the isocratic mode at 0.2 ml/min. The column was set at 35°C. Ivermectin was detected by a Thermo Scientific TSQ Quantum Ultra with HESI-II probe using ESI positive ionization mode; spray voltage of 3000 V; capillary temperature of 200°C; vaporizer temperature of 300°C; sheath gas pressure of 40; aux gas pressure 10; skimmer offset 10 V; SRM setup: Q1: 0.7 FWHM, Q3: 0.7 FWHM, and Q2: 1.5 mTorr (Ar); scan width: 0.002 m/z; scan time at 0.02 s.

2.5. Cimex lectularius Bacterial Gut Cultures. Bed bugs from Test 2 had their gut contents cultured after the morbidity and mortality was recorded on day 64 after ivermectin injection. The healthiest appearing adults were used first, and if no adults were alive, then the healthiest appearing nymphs were used. Bed bugs were surface disinfected to minimize external contaminants by twice vortexing the insects for one minute in a sterile saline solution and pipetting off the fluid. Insects were then exposed to benzalkonium chloride for 1 minute and rinsed again in sterile saline. Rinsates (0.1 mL) from the last washing were spread onto blood agar culture plates to assess the disinfection protocol and found to reproduce no colonies after 72 hours. Bed bugs were thoroughly crushed with a sterile glass rod in 1 ml sterile saline. Serial dilutions were prepared at 10^0, 10^1, and 10^2, and 0.1 ml was spread onto blood agar culture plates (Sigma-Aldrich Inc.) with 2 plates per dilution. Plates were incubated at 25°C for 3 days and then photographed.

2.6. Data Analysis. Comparison of proportions using the "$N-1$" chi-squared test was used to assess for differences

between bed bug morbidity and mortality for those harmed for females, males, and nymphs for each feeding time after injection compared to the control group (Time 0, before injection feeding). Comparison of proportions was used to determine differences in nymphal molting and the number of eggs laid for each feeding time after injection compared to the control group. We used ANOVA to compare group differences in bed bug mortality and incapacitation rates between females, males, and nymphs for feeding times after injection.

3. Results

The morbidity and mortality results are summarized in Tables 1 and 2 (Tests 1 and 2).

3.1. Mortality. Both morbidity and mortality were noted in the 6-hour post-ivermectin injection feeding group in Test 1. After 45 days, the mortality rate was 0/30 (0%), 2/30 (7%), 8/30 (27%), 7/30 (23%), 8/30 (27%), 12/30 (40%), 10/30 (30%), 8/30 (27%), 5/30 (17%), 2/30 (7%), 1/30 (3%), and 3/30 (10%) for the feedings done at 0, 1, 6, 12, 24, 36, 48, 72, 96, 120, 144, and 168 hours, respectively. Peak bed bug mortality occurred at 36 hours after ivermectin injection. The mortality rates for bed bugs fed 6–96 hour after ivermectin injection were significantly different from controls ($p < 0.05$).

Test 2 ivermectin levels in the blood were 0, 2.1, 8.3, 10.4, 12.4, and 18.3 ng/mL for 0, 6, 24, 36, 48, and 72 hours after injection, respectively. The Test 2 day 64 mortality rate was 6/20 (30%), 5/20 (25%), 9/21 (43%), 9/20 (45%), 4/20 (20%), 12/20 (60%), 8/20 (40%), 8/20 (40%), 10/31 (48%), 8/20 (40%), 10/20 (50%), and 8/20 (40%) for the feedings done at 0, 1, 6, 12, 24, 36, 48, 72, 96, 120, 144, and 168 hours, respectively, demonstrating a dose-dependent effect of ivermectin. Only the 36-hour post-ivermectin injection feeding group mortality was significantly different from that of the control group ($p < 0.05$). There was unexpectedly high mortality in all the adult females after feeding making it more difficult to ascertain differences between the experimental groups with the controls.

Adult *C. lectularius* from Tests 1 and 2 showed the highest mortality when fed between 6 and 48 hours after ivermectin injection. Nymph *C. lectularius* from Tests 1 and 2 showed the highest mortality when fed between 12 and 72 hours after ivermectin injection.

3.2. Incapacitation Rate. The incapacitation rate, or the number of insects that were harmed, is the number of bed bugs that were categorized as reactive, immobile, paralyzed, and dead over the number of healthy insects. The Test 1 day 45 incapacitation rate was 0%, 0%, 50%, 55%, 55%, 95%, 100%, 100%, 55%, 45%, 25%, and 20% for the feedings done at 0, 1, 6, 12, 24, 36, 48, 72, 96, 120, 144, and 168 hours, respectively. Peak bed bug harm occurred in the groups fed 36–72 hours after ivermectin injection. All bed bugs that fed after 6 hours after ivermectin injection had significantly more harm than controls ($p < 0.05$). In Test 2 after 64 days, the overall incapacitation rate was 30%, 25%, 43%, 45%, 20%, 60%, 80%, 90%, 90%, 40%, 60%, and 40% for the feedings done at 0, 1, 6, 12, 24, 36, 48, 72, 96, 120, 144, and 168 hours, respectively. Peak bed bug harm occurred in the groups fed 36–96 hours after ivermectin injection where there was significantly more harm than controls ($p < 0.05$). Figure 1 shows the incapacitation rates of Test 1 and Test 2 in hours after ivermectin feeding.

3.3. Insect Paralysis. Ivermectin causes insect paralysis in a dose-dependent manner. However, bed bugs in the same feeding cohort had different responses to the drug after feeding. Some insects that fed on ivermectin-containing blood exhibited no response to light, painful stimuli, sound, and odor, with no apparent cardiac, enteric, or respiratory function that lasted a few weeks. Two males and one female in the 48-hour postinjection feeding cohort were torpid in this state for 21 days before regaining some life functions. In the 72-hour cohort, two males experienced torpor, one experienced some recovery while the other died.

3.4. Refeeding. Test 2 bed bugs were offered blood meals at 16 and 31 days after feeding on the rabbit. Controls and 1- and 6-hour postinjection cohorts fed normally. Some bed bugs, especially those fed 36–72 hours after injection, had difficulty refeeding, and no insects digested their blood meals. It was observed that the cibarial pump, esophagus, and first ventriculus seemed functional, and those that succeeded in skin penetration could not draw blood. Once they had withdrawn, there was no postfeeding defecation indicating a successful feeding. A number of these insects exhibited no control of the maxillary stylus and were physically unable to penetrate the skin. Either the stylus was distally bent, making penetration impossible or force of penetration caused it to loop out between the labrum and maxillary lobe. This led to repeated efforts to feed. Such behaviors as repeated beak grooming, elevated head positions attempting to move the beak into position, running in random circles, and backing up, all were indicative of distress. In the 36-hour postinjection cohort, 15% of the insects attempted to feed and failed; the 48-hour cohort saw no feeding; the 72-hour cohort tried to feed but could not; the 96-hour cohort was mostly nonresponsive and sessile, although a few bed bugs tried to feed and failed; the 120-hour adult cohort did not feed and 20% of nymphs tried but failed; the 144-hour cohort had some nymphs interested in feeding; and the 168-hour cohort initially exhibited disinterest but later all insects fed normally.

3.5. Fecundity. Ivermectin did not affect late-stage preformed eggs in female ovarioles. These eggs were laid and hatched into 1st instar nymphs. Females exposed to sublethal ivermectin doses were often made infertile if oocytes were being formed in the germarium at the time of feeding. Eggs either never developed or were laid without yolk. The few first instar nymphs engendered by sublethal ivermectin-treated females that succeeded to hatch did not appear to be grossly affected and developed into adults.

Table 1: Test 1 data with incapacitation rate for each life stage, exuviae, and eggs at day 45 for each group fed at different hours after ivermectin injection.

Hours	Females ($n = 5$)	Males ($n = 5$)	Nymphs ($n = 10$)	% dead or harmed	Mortality rate (%)	Exuviae	Eggs	Notes
0	5 healthy	5 healthy	10 healthy	0	0	>50	>50	Healthy
1	5 healthy	5 healthy	10 healthy	0 $p = 1.0$	0 $p = 1.0$	>50	>50	
6	5 dead	2 dead, 2 harmed, 1 healthy	1 dead, 9 healthy	50 $p < 0.001$	40 $p = 0.002$	0	0	2 torpid nymphs appeared to recover health
12	5 dead	1 dead, 2 harmed, 2 healthy	2 dead, 1 harmed, 7 healthy	55 $p < 0.001$	40 $p = 0.002$	0	0	Early adult deaths
24	2 dead, 3 healthy	2 dead, 1 harmed, 2 healthy	4 dead, 2 harmed, 4 healthy	55 $p < 0.001$	30 $p = 0.009$	0	0	Early nymph and adult deaths
36	5 dead, 1 healthy	2 dead, 2 harmed, 1 healthy	5 dead, 5 harmed	95 $p < 0.001$	60 $p < 0.001$	1	0	All insects quickly immobile and no blood digestion
48	5 dead	3 dead, 2 harmed	3 dead, 7 harmed	100 $p < 0.001$	55 $p < 0.001$	0	0	High level of harm during first 10 days after feeding, no blood digestion
72	2 dead, 3 harmed	5 harmed	6 dead, 4 harmed	100 $p < 0.001$	40 $p = 0.002$	0	0	Some harmed insects appeared to recover health, no blood digestion
96	1 dead, 4 healthy	1 dead, 2 harmed, 2 healthy	3 dead, 4 harmed, 3 healthy	55 $p < 0.001$	25 $p = 0.02$	0	0	Some harmed insects appeared to recover health
120	3 harmed, 2 healthy	2 harmed, 3 healthy	2 dead, 2 harmed, 6 healthy	45 $p = 0.008$	10 $p = 0.15$	4	5	Most insects appeared harmed and some appeared to recover health
144	1 harmed, 4 healthy	1 harmed, 4 healthy	1 dead, 2 harmed, 7 healthy	25 $p = 0.02$	5 $p = 0.32$	0	0	Most population appeared to recover health
168	1 harmed, 4 healthy	1 dead, 4 healthy	2 dead, 8 healthy	20 $p = 0.04$	15 $p = 0.08$	0	0	Population appears mostly healthy

Table 2: Test 2 data with each life stage recorded as dead (D), incapacitated (I), or healthy (H) at day 64 after feeding. Also recorded are the breath test, feeding 1 test, feeding 2 test, exuviae, and eggs at day 64 for each group fed hours (Hr) after ivermectin injection.

Hours	IVM level (ng/mL)	Females ($n = 5$)	Males ($n = 5$)	Nymphs ($n = 10$)	% dead or harmed	Mortality rate (%)	Breath test day 14	Feeding 1 on day 16	Feeding 2 on day 31	Exuviae	Eggs
0	0	5 D	1 D, 4 H	10 H	30	30	Normal	Normal	Normal	>50	>50 (>50 new nymphs)
1		5 D	5 H	10 H	25 ($p = 0.73$)	25 ($p = 0.73$)	Normal	Normal	Normal	15	45 (27 new nymphs)
6	2.1	5 D	2 D, 3H	2 D*, 9H	43 ($p = 0.39$)	43 ($p = 0.39$)	Low interest	None	Normal	11	23 (13 new nymphs)
12		5 D	2D, 3 H	2 D, 8 H	45 ($p = 0.33$)	45 ($p = 0.33$)	Low interest	Low interest	Low interest	9	12 (9 new nymphs)
24	8.3	1 D, 4 H	1 D, 4 H	2 D, 8 H	20 ($p = 0.47$)	20 ($p = 0.47$)	Low and no interest	Low and no interest	Normal	4	0
36		5 D	5 D	2 D, 8 H	55 ($p = 0.11$)	55 ($p = 0.11$)	Low and no interest	Low and no interest	Failed feeding attempt and low interest	3	13 (8 new nymphs)
48	12.4	5 D	3 D, 2 I	6 I, 4 H	80 ($p = 0.002$)	40 ($p = 0.51$)	No interest	No interest	No interest	3	0
72	18.3	3 D, 1 I, 1 H	4 D, 1 I	1 D, 8 I, 1 H	90 ($p < 0.001$)	40 ($p = 0.51$)	Low and no interest	None	Failed feeding attempt	7	10 (4 new nymphs)

Table 2: Continued.

Hours	IVM level (ng/mL)	Females ($n = 5$)	Males ($n = 5$)	Nymphs ($n = 10$)	% dead or harmed	Mortality rate (%)	Breath test day 14	Feeding 1 on day 16	Feeding 2 on day 31	Exuviae	Eggs
96		4 D, I, H	4 D, 1 I	2 D*, 9 I	90 ($p < 0.001$)	50 ($p = 0.2$)	No interest	No interest	Failed feeding attempt and low interest	5	9 (8 new nymphs)
120		4 D, 1 H	3 D, 2 H	1 D, 9 H	40 ($p = 0.51$)	40 ($p = 0.51$)	No interest	No interest	Failed feeding attempt and low interest	6	3 (0 new nymphs)
144		5 D	3 D, 2 I	2 D, 8 H	60 ($p = 0.06$)	50 ($p = 0.2$)	No interest	Failed feeding attempt and low interest	Failed feeding attempt and low interest	5	10 (9 new nymphs)
168		5 D	2 D, 3 H	1 D, 9 H	40 ($p = 0.51$)	40 ($p = 0.51$)	Normal	Normal	Failed feeding attempt and low interest	5	27 (25 new nymphs)

*11 nymphs were fed.

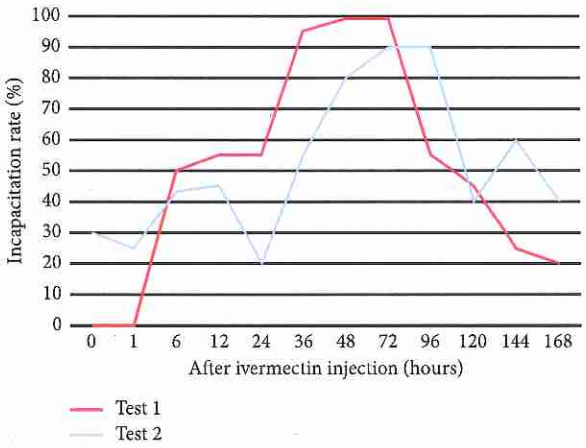

Figure 1: Bed bug incapacitation rates after feeding on a rabbit injected with 0.3 mg/kg of ivermectin (Test 1 and Test 2).

Ivermectin had a dramatic and long-term decrease in both the number of eggs that were laid and the resulting new instars. In Test 1, there were >50 eggs laid in the control group, and only 5 eggs laid for all other feeding cohorts. In Test 2, there were >50 eggs laid in the control group, and 45, 23, 12, 0, 13, 0, 10, 9, 3, 10, and 27 eggs laid in the 1, 6, 12, 24, 36, 48, 72, 96, 120, 144, and 168 hours after ivermectin injection groups.

3.6. Molting. Nymphal molting was significantly reduced for bed bugs that fed between 6 and 168 hours after injection (Figure 2). For Test 2, all nymphs were offered two blood meals and thus the opportunity to molt twice. By the end of the 64-day observation period for Test 2, high numbers of exuviae were present in the controls because 5th instar females matured during the test period and engendered many 1st and 2nd instar nymphs. However, all Test 2 postinjection

Figure 2: Nymph showing incomplete molt after taking an ivermectin blood meal.

(a)

(b)

FIGURE 3: Agar plates of bed bug gut cultures from Test 2 (a) nymphs and (b) adults >64 days after feeding on the rabbit observed at 72 hours.

cohorts experienced suppression of nymphal development at day 64.

3.7. Cimex lectularius Gut Cultures. We found that bed bugs that fed on the rabbit between 36 and 96 hours after ivermectin injection had no microbial growth on blood agar plates. After 96 hour, bacterial growth was again observed. The growth plates are shown in Figure 3.

4. Discussion

We show that bed bugs which take an *in vivo* blood meal containing ivermectin suffer dose-dependent toxicity

including death, reductions in fecundity, inability and disinterest in refeeding, and molting. Ivermectin blood concentrations of ~2 ng/mL in the rabbit were required to see changes in bed bug fecundity, and concentrations of at least 8 ng/mL were required before other significant bed bug toxicity was observed. Our results are congruent with reports investigating ivermectin and bed bugs where morbidity and mortality was noted between 2.5 and 25 ng/mL using an *in vitro* feeding system [9, 10]. Ivermectin also alters the *C. lectularius* gut microbiome—the implications of which require further investigation.

There are limited *in vivo* data demonstrating the toxicity of ivermectin against bed bugs and no studies where ivermectin blood levels were measured. A small group of bed bugs were fed on mice after receiving high doses of ivermectin intraperitoneally, which resulted in high insect morbidity and mortality [8]. Bed bugs were also allowed to feed on four human volunteers given 0.2 mg/kg of oral ivermectin, which caused insect morbidity and mortality in a dose-dependent manner [8]. Limitations of these studies include that only a few bed bugs fed on each human subject, the insects were observed for less than a month, not all life stages were evaluated, the bed bugs were fed soon after the administration of ivermectin, and bed bugs were only reported as dead or harmed and did not account for other manifestations of morbidity such as reductions in fecundity or the ability to refeed.

In our experiments, we found that peak bed bug morbidity and mortality occurred when insects fed 36 to 72–96 hours after ivermectin injection. We were unable to obtain blood samples after 72 hours after ivermectin injection, and we are unsure if the peak serum concentration of ivermectin occurred between the 48–72 hour sample or after the 72-hour sample. Based on our bed bug morbidity and mortality results and the previously reported pharmacokinetics of subcutaneous ivermectin in a rabbit, we suspect the peak plasma concentration in the rabbit occurred around 72 hours after injection [14, 15]. The pharmacokinetics of subcutaneously administered ivermectin in a rabbit does not mirror the pharmacokinetics of orally administered ivermectin in humans which reaches a peak plasma concentration in about four hours [11].

There have not been any human clinical trials using ivermectin for bed bug control. Our data show that ivermectin may not need to kill all bed bugs in the population in order to eliminate an infestation because the ivermectin greatly reduces fecundity, prevents nymphal molting, and inhibits insect refeeding. Ivermectin could potentially be used as an adjunctive therapy for humans with bed bugs along with traditional integrated pest management (IPM) practices.

5. Conclusions

Ivermectin causes dose-dependent toxicity in *C. lectularius* when fed on a rabbit injected with ivermectin. Ivermectin blood levels of ~2 ng/mL cause changes in bed bug fecundity, and levels of >8 ng/mL are required for significant bed bug mortality. Ivermectin causes alterations in the bed bug gut microbiome.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

The present address of Johnathan M. Sheele is Mayo Clinic, Department of Emergency Medicine, 4500 San Pablo Road, Jacksonville, FL 32224, USA.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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RESISTANCE TO DEHYDRATION BETWEEN BOUTS OF BLOOD FEEDING IN THE BED BUG, *CIMEX LECTULARIUS*, IS ENHANCED BY WATER CONSERVATION, AGGREGATION, AND QUIESCENCE

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Abstract. To determine how the bed bug, *Cimex lectularius*, survives in a dry environment for many months without feeding, water-balance characteristics were compared for all stages from first-instar nymphs to adults. This species is characterized by a low net transpiration rate averaging < 0.2%/h, high tolerance for dehydration (30–40% loss in body water), and an impermeable cuticle as indicated by a high critical transition temperature (CTT) in the 35–40°C range, implying that this insect is adapted for desiccation-hardiness. The capacity of adults to survive for 2 weeks at $0.00a_v$ (a_v = % RH/100) with no access to food or water exemplifies this trait. In contrast to more mature stages, first-instar nymphs contain more water, lose water at a faster rate, experience abrupt water loss at a lower temperature, and survive less time in dry air, suggesting that this stage is the most sensitive to water stress. This insect relies on blood to replenish water stores; none of the stages examined have the capacity to absorb water vapor (critical equilibrium activity, CEA $\geq 0.99a_v$), and they drank only sparingly when offered free water. As the bed bugs progress through their development, they gradually reduce their water requirements while increasing their desiccation resistance. Surviving water stress is considerably enhanced behaviorally by quiescence, characterized by prolonged periods of inactivity, and by the formation of clusters that generate a water-conserving group effect.

INTRODUCTION

The common bed bug, *Cimex lectularius*, has a remarkable ability to survive 4 months to 2 years without feeding,[1] a feature that presumably accounts for their incredible capacity to persist for long periods in human bedding and other locations. Recently, there has been a resurgence of bed bugs in human dwellings, including hotels.[2–4] The prolonged absence of a suitable host is problematic for these obligate blood feeders due to a lack of fluid uptake to counter desiccation.[5,6] How do bed bugs cope with this challenge? Besides cannibalism,[1] no other attributes have been described that would account for their impressive survival capacity between bouts of blood feeding. Despite the current bed bug epidemic, few studies, with the exception of earlier works by Johnson[5,6] and those summarized in Usinger,[1] have focused on the physiology of bed bugs, especially their mechanisms for enduring prolonged periods of fasting.

Epidemiologically, *C. lectularius* is a nuisance pest to humans, causing loss of sleep due to annoying bites.[2–4] The bed bug does not appear to be a disease vector, but it has been associated with iron deficiency, secondary bacterial infection from bite sores, and allergic hypersensitivity.[7–9] This species is broadly distributed, occurring throughout much of the temperate zone, and is most successful in cosmopolitan areas with high human density.[1] All five nymphal stages and adults are mobile and require a blood meal to molt, and the adult requires an additional blood meal to reproduce.[2] Bed bugs remain hidden during the day, and because of their small size, lack of wings, and flat body shape, they are able to crawl into tight crevices. After feeding forays, the bugs return to these sites, resulting in the formation of dense aggregations (mixed stages), known as "brood centers," where eggs, fecal material, and exuviae also accumulate.[1,2] When in these clusters, the bugs enter a quiescent state while the blood meal is digested; they venture out again only for host seeking when their metabolic reserves have been depleted.[1,10,11]

Previous research, discussed in a review by Johnson,[5] on the water requirements of *C. lectularius* indicates that this bug is particularly tolerant of drying,[1,5,6] but factors influencing their unique dehydration resistance and how these factors may contribute to the recent proliferation of bed bugs have not been determined. In this study, we construct a water-balance profile for *C. lectularius* with the goal of examining habitat preference and suitability, features that are critical for survival and for determining the bug's potential to spread into new regions. We examine the entire life cycle (except eggs), using bugs of similar age to illustrate developmental shifts in water requirements and to pinpoint the stages that are most and least vulnerable to water stress. We assess percentage body water content, dehydration tolerance limit, net transpiration rate (integumental plus respiratory water loss), critical transition temperature (CTT, denoting the temperature threshold of an abrupt lethal water loss), and free water drinking ability. The benefit of clustering for water conservation was evaluated by measuring net transpiration rates of individual bugs and of different-sized groups. Rivnay[12] and Johnson[6] showed prolonged survival of various bed bug stages at conditions of water deficiency that are similar to those regularly encountered in human dwellings (30–50% RH and 22–24°C based on comfort standards),[13,14] thus suggesting that these bugs may have the capacity to absorb water vapor from the air. An additional goal of our study was to determine the bed bug's critical equilibrium activity (CEA), to test if the bugs can use atmospheric moisture as a primary source of water.

MATERIALS AND METHODS

Bed bugs and test conditions. *C. lectularius* was acquired from The Ohio State University Insectary. The colony was established in 2002 from individuals collected in Columbus, OH. Bugs were stored at 85% RH, 15 h:9 h light/dark until

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they were used for these experiments. Temperature was 25 ± 1°C for the colony and also for basic observations; this temperature allows us to compare our results with previous studies on insect water balance.[15] Each of the five nymphal stages and male and female adults were used in the experiments. All individuals were used 1 week after molting or after hatching in the case of the first instar. An aspirator and felt-tipped soft forceps were used for transferring the bugs and for handling the bed bugs during mass measurements.

Test relative humidities (% RH) were generated with the use of saturated salt solutions (33% RH with $MgCl_2$, 75% with NaCl, 85% with KCl, 93% with KNO_3, 98% with K_2SO_4) as described by Winston and Bates,[16] distilled water (100% RH) or calcium sulfate ($CaSO_4$, 0% RH; 1.5×10^{-2} % RH)[14] that was placed in the base of sealed glass desiccators (5,000 cc). A hygrometer (SD ± 0.5% RH; Thomas Scientific, Philadelphia, PA) was used to verify each experimental relative humidity. To relate the water present within the bugs to that in the surrounding atmosphere, relative humidities were expressed as water vapor activities (a_v; a_v = % RH/100; thus, $0.00a_v$, $0.33a_v$, $0.85a_v$, $0.93a_v$, $0.98a_v$ and $1.00a_v$) and the activity of the body water (a_w) of the bug = $0.99a_w$ based upon mole fraction.[17] Bed bugs were housed individually within 1-cc mesh-covered chambers that were placed on perforated porcelain plates to prevent contact with the solution used to generate the water-vapor activities.

Bugs were weighed individually using an electrobalance (CAHN; SD ± 0.2 μg precision and ± 6 μg accuracy at 1 mg; Ventron Co., Cerritos, CA) without enclosures and without the use of anesthesia. Briefly, a bug was removed from its enclosure and permitted to crawl onto the weighing pan of the balance, the mass was determined, and the bug was picked up with an aspirator and returned to the 1-cc mesh enclosure and test conditions. This was accomplished in < 1 minute. Before being used in experiments, the bugs were held at $0.33a_v$ until a 4–6% loss in body weight occurred, thus minimizing the effect of excretion, digestion, and reproduction on mass changes[18,19] and standardizing the bugs with regard to water flux so that mass changes only reflected internal fluctuations of the bug's water content.[20] At the end of each experiment, bugs were placed at 90 °C and $0.00a_v$ and monitored until mass became constant, then held for an additional 3 days of drying; this mass was then recorded as the dry mass.

Water-balance characteristics. Wharton's methods[18] and equations, with modifications by Yoder and Spielman,[21] Kahl and Alidousti,[22] and Benoit and others,[20] were used to determine the various water balance characteristics. Dry mass (d) was subtracted from initial (fresh) mass (f) to determine the amount of water that is available for exchange, which is defined as the water mass (m). Water mass was expressed as a percentage of the initial mass to determine the percentage body water content. Bugs were placed at $0.33a_v$ and 30°C, weighed every hour, and tested for their ability to right themselves and crawl five body lengths. The mass measurement that corresponded to the point where they were unable to achieve this behavioral task was defined (after subtracting corresponding dry mass) as the critical mass, m_c, and was used as an estimate of dehydration tolerance based on the percentage change in mass lost from initial to critical mass (m_c).

To determine net transpiration rate (= integumental plus respiratory water loss), bugs were weighed, placed at $0.00a_v$, and reweighed at various intervals, for a total of five readings of mass; weighing intervals varied between instars depending on the extent of their desiccation. Net transpiration rate was determined at $0.00a_v$ because the amount of water mass lost declines exponentially such that water loss rate can be derived from the slope of a line described by the equation: $m_t = m_0\exp(-k_t)$, where m_t, is the water mass at any time t, m_0 is the initial water mass, and $-k_t$ is the rate of transpiration expressed as %/h. Rates were established for isolated individuals and also for individuals in groups of different sizes. Individuals were marked on the dorsum with a spot of paint (Pactra, Van Nuys, CA) and allowed to cluster naturally. The paint-marked bugs were removed for mass determinations and then returned to the group; paint had no effect on mass changes (data not shown). Critical transition temperature (CTT), the temperature threshold of a rapid water loss, was based on change in activation energy (E_a) determined by analyzing water-loss rates over a broad temperature range (4–60°C) as described by the Arrhenius equation: $k = A\exp[-E_a/(RT)]$, where k is the net transpiration rate, A is steric (frequency) factor, T is absolute temperature, R is gas constant, with E_a based on the slope, which is equal to $-E_a/R$.

Avenues of water gain were also investigated. Drinking free water was analyzed by offering bugs 5- to 20-μL droplets of 0.5% Evans blue-stained water in a 100 × 15 mm petri dish (0.75% RH, 25°C). Ten bugs were placed in each dish, examined for 20 minutes (40× microscopy), and then every 2 hours thereafter. After 24 hours of observation, bugs were dissected in 0.1% NaCl under the microscope (100×) and examined for the presence of blue coloration in their digestive tract and liberation of dye when the gut was opened. Water vapor absorption was examined by long-term daily monitoring of water mass (m) of individual bugs held at different water-vapor activities. The capacity to maintain a steady water mass in subsaturated air (< $0.99a_w$ of the bug's body water), thus balancing water loss with water gain from the air (water gain = water loss), was taken as evidence of the bug's ability to use atmospheric water vapor as a primary source of water. Survivorship was assessed based on 40× microscopic observations of dead bugs; bugs were considered dead if they were immobile, unable to right and crawl, and failed to respond to prodding or bright light.

Sample sizes and statistics. Each experiment was replicated 3 times with 10 bed bugs per replicate, for a total of 30 individuals for each water-balance characteristic determination. Data, reported as the mean ± SE, were compared with analysis of variance (ANOVA), and arcsin transformation was used in the case of percentages.[23] A test for the equality of slopes of several regressions was used to compare characteristics derived from regression lines. Survivorship times were compared using t statistics.

RESULTS

Water content. Initial mass, dry mass, and water mass increased with each successive stage (Table 1). Percentage body water content was highest for first-instar nymphs (71%) and lowest (67%) for female adults (ANOVA; $P < 0.05$). No significant difference in percentage body water content was observed between sexes (ANOVA; $P > 0.05$). In all cases, within a particular stage, the water mass was a positive correlate of dry mass, with $R \geq 0.93, 0.89, 0.94, 0.93, 0.95$ for first- through

TABLE 1
Comparison of water balance characteristics for different stages of *Cimex lectularius**

Characteristics	Nymphs					Adults	
	1st	2nd	3rd	4rh	5th	Male	Female
Water content							
Initial mass, f (mg)	0.124 ± 0.023	0.255 ± 0.016	0.821 ± 0.037	1.323 ± 0.041	2.621 ± 0.044	4.892 ± 0.037	5.472 ± 0.046
Dry mass, d (mg)	0.37 ± 0.009	0.077 ± 0.011	0.250 ± 0.021	0.408 ± 0.031	0.819 ± 0.032	1.561 ± 0.029	1.805 ± 0.032
Water mass, m (mg)	0.087 ± 0.010	0.178 ± 0.021	0.571 ± 0.033	0.912 ± 0.036	1.802 ± 0.034	3.331 ± 0.041	3.677 ± 0.052
Water content (%)	70.9 ± 2.1	69.8 ± 1.7	69.5 ± 1.4	69.1 ± 1.9	68.8 ± 2.2	68.1 ± 1.8	67.2 ± 2.6
Water loss							
NTR (%/h; Figure 1)	0.402 ± 0.011	0.322 ± 0.019	0.181 ± 0.007	0.152 ± 0.018	0.121 ± 0.009	0.101 ± 0.007	0.091 ± 0.011
m_c (mg)	0.054 ± 0.004	0.112 ± 0.011	0.364 ± 0.016	0.589 ± 0.026	1.196 ± 0.031	2.241 ± 0.021	2.394 ± 0.036
DT (%)	37.4 ± 4.6	36.8 ± 4.1	36.2 ± 2.8	35.4 ± 2.9	33.6 ± 2.6	32.9 ± 0.9	34.9 ± 1.5
Survivorship at $0.00a_v$	3.9 ± 0.9	4.8 ± 0.6	8.3 ± 1.1	9.8 ± 1.2	11.6 ± 0.6	13.6 ± 0.8	16.0 ± 1.5
CTT (°C; Figure 3)	34.5 ± 2.4	35.9 ± 2.2	36.7 ± 1.9	37.6 ± 2.2	38.2 ± 1.6	38.9 ± 2.0	39.2 ± 1.1
Water gain							
Free water uptake	+	+	+	+	+	+	+
CEA (Figure 4)	$\geq 0.99a_v$	$\geq 0.99a_v$	$\geq 0.99a_v$	$\geq 0.99a_v$	$\geq 0.99a_v$	$\geq 0.99a_v$	$\geq 0.99a_v$

* f, fresh (initial mass); d, dry mass; m, water mass; %, percentage body water content; m_c, critical water mass where they were unable to right and crawl; NTR, net transpiration rate; DT, dehydration tolerance as percentage lost to m_c; CTT, critical transition temperature; survivorship is based on 50% of the bugs; drinking confirmed by presence of blue tracer in water droplet upon dissection; and CEA (critical equilibrium activity), the range of water vapor activities (a_v = % RH/100) where water vapor absorption occurs. Data shown are means ± SE, n = 30.

fifth-instar nymphs, respectively, 0.96 for adult males and 0.95 for adult females (ANOVA; $P < 0.001$). Corresponding water mass to dry mass values through the series of first- through fifth-instar nymphs were 2.35, 2.31, 2.28, 2.24, and 2.20, respectively, showing a progressive decline as dry mass increased in proportion to water mass. Water mass to dry mass ratios for adults were 2.13 for males and 2.04 for females. Within a particular stage, individuals were similar in size and shape and water content, indicating that surface area to volume properties are standardized; thus each stage can be anticipated to exhibit a consistent, stage-related water flux. We conclude that the proportion of body water is highest for bed bugs in their earliest stages of development.

Net transpiration rate. First-instar nymphs lost water (net transpiration rate) at a rate of 0.402 ± 0.011%/h (Table 1). The corresponding rate of water loss for individuals (paint marked) in a group of 20 (0.247 ± 0.010%/h) was approximately half that of isolated individuals (Table 1; Figure 1; ANOVA; $P < 0.05$). Net transpiration rates of first-instar nymphs in groups of 5 (0.360 ± 0.014%/h) and 10 (0.311 ± 0.017%/h) were between these extremes (ANOVA; $P < 0.05$). In all cases, the paint-marked individuals were returned to the group after mass determination, and they remained in the group. It is also important to note that members of the groups did not disband when disturbed by removing or reintroducing the paint-marked individuals and individuals were in direct contact with neighboring bugs. Periodically, the location of the bed bugs was recorded and movement was noted; these observations suggested that individuals likely spend nearly equal time in the middle and at the edges of the group. Net transpiration rates of other stages are presented in Table 1 and follow a similar exponential pattern of water loss ($R >$ 0.99; ANOVA; $P < 0.001$), reflecting proportionate loss at $0.00a_v$ on a semilogarithmic plot as illustrated by first-instar nymphs in Figure 1. Net transpiration rate increased with each successive stage during development (Table 1; ANOVA; $P < 0.05$). Net transpiration rate also correlated with dry mass ($y = -0.38x$, $R = 0.94$; ANOVA; $P < 0.001$; Figure 2), indicating that water loss varies according to body size. Net transpiration rate was 4× higher for first-instar nymphs than adult females, a stage that is nearly 50× larger (Table 1). We conclude that water is lost most rapidly when the surface area is greatest relative to volume and that a strong, positive relationship exists between aggregation size and suppression of water loss.

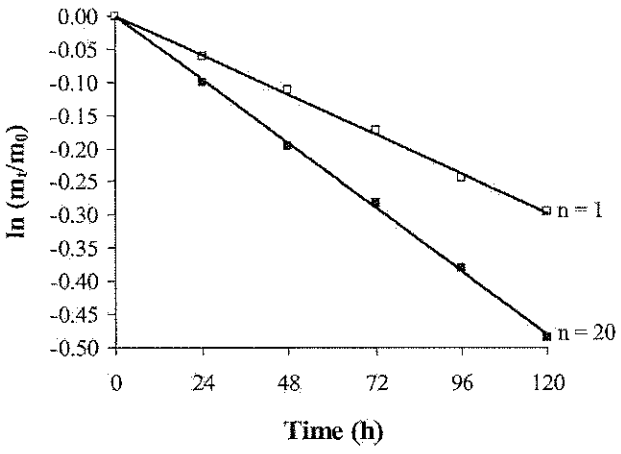

FIGURE 1. Proportion of water mass lost at $0.00a_v$ (a_v = % RH/100) and 25°C in first-instar nymphs of *C. lectularius* as single isolated individuals and as an individual in a group of 20 first instars. The slope of the regression line is the rate of water loss (= net transpiration rate, integumental plus respiratory water loss). m_t, water mass at any time t; m_0, initial water mass.

Critical transition temperature. Net transpiration rate of first-instar nymphs increased with increasing temperature and exhibited a Boltzmann temperature function ($R > 0.95$; ANOVA; $P < 0.001$; Figure 3). A distinct critical transition temperature (CTT) was detected in these first-instar nymphs as evidenced by a steep slope of the regression line indicative of a new temperature range (biphasic, two-component curve) and a change in activation energy (E_a). The change in slope was due to higher proportionate amounts of water loss in the higher temperature range. The CTT of first-instar nymphs was 34.5°C, as determined by identifying the point of intersection of the two regression lines plotting the E_a changes. Other stages responded similarly to temperature and yielded nearly identical net transpiration rate–temperature relation-

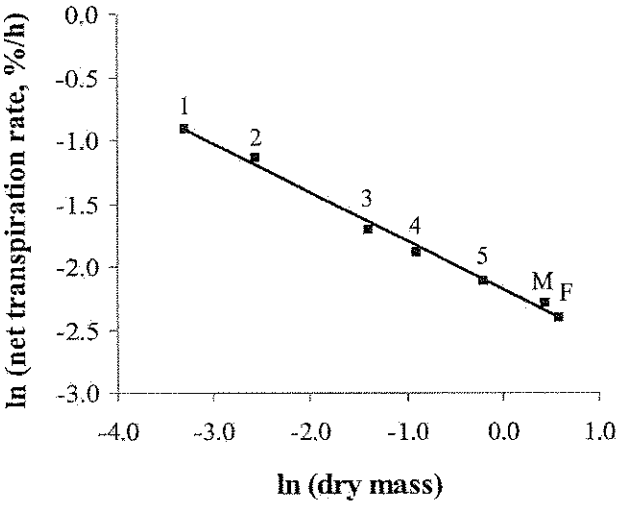

FIGURE 2. Relationship between net transpiration rate (water loss rate) and body size (dry mass) in stages of *C. lectularius*. Numbers above symbols on graph correspond to the various nymphal stages; M, male; F, female. Note high degree of fit of the regression line to a straight line ($R = 1.00$).

ships as shown for first-instar nymphs in Figure 3; the results indicate Boltzmann dependence on temperature ($R > 0.95$; ANOVA; $P < 0.001$), different proportionate water mass losses in low and high temperature ranges leading to a change in slope, and evidence for a CTT. The CTT increased through development from 34.5°C for first-instar nymphs to 39°C for female adults (Table 1; ANOVA; $P < 0.05$). Compared with adults, earlier stages are more at risk of abrupt, rapid desiccation at high temperature.

Dehydration tolerance. When examined under the microscope (40×), bed bugs remained immobile and failed to respond to prodding for 15–20 seconds, then slowly uncurled

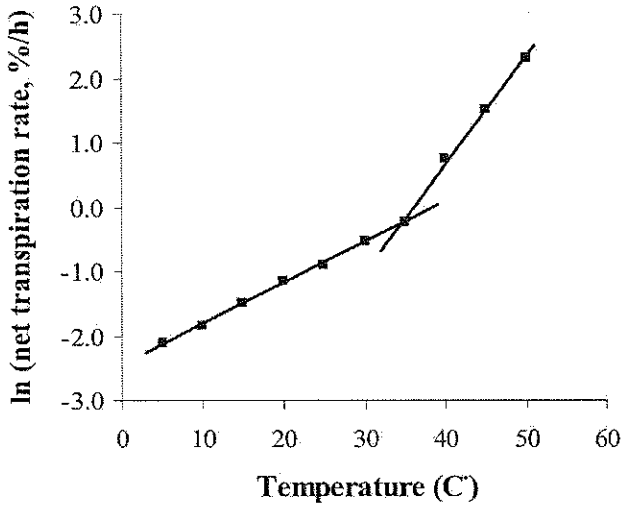

FIGURE 3. Water retention (net transpiration rate) as a function of temperature in first-instar nymphs of *C. lectularius*. Critical transition temperature (CTT), where water loss increases abruptly, is detected indicated by the point of intersection denoting change in slope of the regression lines (two-component curve) corresponding to a change in the activation energy (E_a) for water loss.

their legs, righted, and began to crawl. Once having lost about ⅓ of their water content, they failed to right themselves and crawl 10 body lengths, as indicated by critical mass (m_C) values (Table 1). No significant differences were observed among the various stages when critical mass was expressed as a percentage of the amount of body water that was lost, averaging 35% (Table 1; ANOVA; $P > 0.05$). Throughout their life cycle, bed bugs tolerated similar levels of dehydration stress.

Survivorship. Female adults were capable of surviving a remarkable 16 days (50% of adults) at $0.00a_v$ with no food or water, demonstrating their ability to withstand prolonged periods of starvation and desiccation. Females survived approximately 2 days longer than males (t statistics; $P < 0.05$). These survivorship estimates for adults agree well with our calculated dehydration tolerance limits and net transpiration rates (Table 1). Similarly, the time required to reach critical mass (dehydration tolerance limit) calculated from net transpiration rates for each of the immatures closely matched length of survival in dry air (Table 1; t statistics; $P < 0.05$). Thus, the relationship between net transpiration rate, dehydration tolerance, and length of survival in dry air are consistent for all stages throughout the life cycle. Once they reached their critical mass, the bugs were unable to be rescued by placing them at $1.00a_v$ or by offering them droplets of free water (each $N = 10$/stage), thus indicating that they had sustained an irreversible level of dehydration. None of the bugs in this condition survived. Our results show that adults are more resistant to desiccation than immatures.

Free water drinking. When bed bugs were placed into a petri dish containing droplets of Evans blue-stained water, they crawled about quite actively as though engaged in a searching type of behavior, characterized by tapping the bottom of the dish with the antennae as they moved about the arena. Within 1–2 minutes, most of the movement stopped and the bed bugs were observed to be clustered around the edges of the dish. Little movement was observed within these aggregations or to adjacent clusters. In all cases, water droplets were encountered passively, and none of the bugs displayed deliberate movements away or toward the droplets of water. They did not appear to be attracted or repelled by the droplets. On occasion, upon encountering a droplet of water, the bug paused, inserted its proboscis into the droplet and drank; this lasted for approximately 1 minute (Table 1). This set of behavioral observations included each of the various stages examined. Blue dye could be seen filling the gut diverticula when the bug was observed under the microscope (40×), and blue coloration was liberated from the gut upon dissection (100×), a confirmation that the bugs consumed the colored liquid. Attempts to encourage the bugs to drink by dehydrating them by losses of 20–25% of their body mass ($0.00a_v$ and 30°C) prior to exposing them to the water droplets were futile. Instead, these dehydrated bed bugs formed clusters and only a few were observed to imbibe the water and the water droplets were still encountered passively; that is, no deliberate movements were made to the droplets. Although bed bugs have the ability to drink water, they appear to do so rather sparingly.

Water vapor absorption. First-instar nymphs failed to maintain their water mass over a period of several days when exposed to subsaturated air (Figure 4). This was due to the activity gradient created between the activity of the body wa-

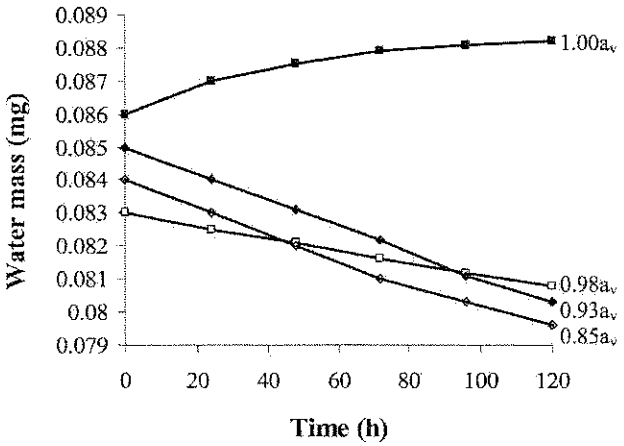

FIGURE 4. Changes in water mass of first-instar nymphs of *C. lectularius* in response to different water vapor activities (a_v; a_v = % RH/100) and 25°C. Inability to maintain a steady water mass in dynamic equilibrium (water gain ≠ water loss) in subsaturated air indicates that they do not actively absorb water vapor from the air.

ter ($0.99a_w$[18]) with that of surrounding air, thus $0.99a_w > 0.98a_v$, $0.93a_v$, $0.85a_v$, and $0.75a_v$, and this resulted in water loss by simple diffusion. Less net water loss was measured as the air became more humid ($R > 0.97$; ANOVA; $P < 0.001$), which demonstrated an effect of greater passive adsorption onto the surface of the cuticle as water vapor activity approached saturation. Water gain occurred and the uptake was maintained in saturated air of $1.00a_v$ because the activity gradient was reversed, $1.00a_v > 0.99a_w$ (body water), and this drove water inward into the bug's body and contributed to water mass. Because net water loss occurred at $0.98a_v$ and net water gain at $1.00a_v$, water balance was achieved (gain = loss) at $0.99a_v$, which is equivalent to the $0.99a_w$ body water activity estimated by Wharton.[18] Accordingly, the critical equilibrium activity (CEA) of first-instar nymphs was $\geq 0.99a_v$. Other stages displayed a similar pattern of water loss in subsaturated air and gain at saturation consistent with properties of diffusion as illustrated by Figure 4 for the first-instar nymph. For all stages, CEA $\geq 0.99a_v$ (Table 1). Thus, bed bugs cannot use water vapor as a significant source of water.

DISCUSSION

Outstanding water balance features of *C. lectularius* include a low net transpiration rate (< 0.2%/h) that is similar to rates observed in desert-adapted arthropods, an ability to tolerate loss of ⅓ of their body water (most arthropods only tolerate 20–30% loss), and a high (> 35°C) critical transition temperature consistent with arthropods that have water impermeable cuticles.[15,18,24] This set of water-balance characteristics is typical for arthropods that are differentially adapted for life in a dry habitat according to Hadley.[15] Thus, *C. lectularius* is xerophilic with regard to water balance. As such, *C. lectularius* is modified for water conservation and to resist desiccation, wherein their ability to retain water (low water loss rate) is more important than their ability to gain water. Other water-balance characteristics of *C. lectularius*, such as the ≈ 69% body water content, free water drinking, and lack of ability for water vapor absorption are similar to features observed in most arthropods.[15] In fact, as adults, the characteristics of *C. lectularius* resemble those of another blood-feeding hemipteran, *Rhodnius prolixus* (69% water content, 50% dehydration tolerance, low water loss, xeric ecologic classification).[15,25,26] Notably, emphasis on water retention and desiccation-resistance properties by *C. lectularius* enables this species to function effectively in a dry environment and dually protects the bugs against desiccation between blood meals or in the absence of a host.

The fact that *C. lectularius* failed to absorb water vapor is not unusual, as most arthropods lack this ability.[15,17] Arthropods that achieve this feat maintain body water levels at otherwise dehydrating conditions by absorbing water against the atmospheric gradient using a mechanism frequently involving a salt-driven process.[27,28] Because water vapor absorption does not occur in *C. lectularius*, the long-term survivorship in the absence of food and in relatively dry air, reported by Rivnay[12] and Johnson,[6] must be the consequence of water conservation properties rather than the ability to use water vapor from the air. For all stages, the CEA $\geq 0.99a_v$, indicating that water gain can only occur from air that is completely water-saturated at $1.00a_v$, a water vapor activity that corresponds to water as a liquid. This necessarily implies that water must be imbibed in fluid form.[19] In the presence of free water, however, stages of *C. lectularius* were not attracted to droplets of water and only drank on occasion, displaying no real interest in the water even when they were dehydrated. Such inconsistent drinking patterns were similarly observed in certain ticks[22] and are typical of arthropods that feed on blood.[15] Indeed, blood feeding is responsible for the majority of the contributions to the internal body water content of *C. lectularius*,[11] as well as *R. prolixus*,[29] a species that shares many water-balance characteristics with *C. lectularius*. Although free water represents a viable water resource, the most likely primary source of water for *C. lectularius* is their blood diet.

Other noteworthy features related to water balance in *C. lectularius* are primarily behavioral regulators of water loss due to the capacity to enter long-term quiescence and the ability to form clusters. The quiescence is characterized by a complete lack of activity, including retraction of the legs that give the bugs a dead appearance. This quiescence can only be broken by persistent prodding and provoking. Clearly, this shut-down makes a major contribution to conservation of the body water content by helping to reduce respiratory water loss,[15] an ability that is similar to that seen in the spider beetle, *Mezum affine*, a species that can survive without water for many months.[20] Suppression of net transpiration rate is common in arthropods that aggregate, including mites,[30] beetles,[31] and cockroaches.[32] A well-known additional benefit of aggregation is to increase access to mates,[31] and this is consistent with the designation of the bed bug cluster as a "brood center."[1] Although our experiments on the group effect were restricted to first-instar nymphs, it is likely that all stages experience and benefit from this effect. Our preliminary results using flowing dry air suggest that the mechanism of the group effect operates by generating a humidified boundary layer, as noted in beetles.[33] Linkage between quiescence and enhanced water conservation is similar to that observed during overwintering diapause.[34] This feature raises the yet untested possibility that quiescent stages of *C. lectularius* may also be cold tolerant.

Developmentally, the stage of *C. lectularius* that is most

sensitive to desiccation stress is the first-instar nymph. This stage has the highest percentage body water content, indicating that it requires more body water to function. The first-instar nymph is also the smallest in body size, thus making it more vulnerable to rapid water loss rate due to its surface area to volume properties. Greater cuticular permeability to water is implied by a lower CTT, suggesting that the cuticular structure of the first instar is more easily disrupted. The high water requirement must be met by increased feeding activity or a greater reliance on clustering. Stage differences in water requirements are unlikely to be indicators of a different habitat preference because all instars are found clumped together within the same microhabitat.[1] Through development, percentage body water content declines concurrently with an increase in dry mass (= fat)[13]; thus, the bugs require less water and accumulate greater fat reserves as they become older. In addition, as the bed bugs proceed through development, they increase in body mass (size), allowing for greater water retention, and the CTT increases by 5°C, implying that the integument becomes progressively more water-tight. Thus, there is a gradual shift through development from a high to a low water requirement, and this is reflected by differences in survival enabling adults to live the longest in a desiccating environment.

From this study it is clear that the water balance strategy of *C. lectularius* emphasizes water retention. These effective water-conserving traits are supplemented behaviorally by a quiescence marked by periods of inactivity and a group effect that enhances protection against desiccation stress. Effective water conservation thus enables the bed bugs to persist for long periods without rehydration. It would appear that the human comfort standards of 0.30–0.50a_v and 22–24°C[13,14] create an ideal habitat for *C. lectularius*. As long as a host is available on occasion for blood feeding, the species is well adapted to survive the water balance challenges encountered in most human dwellings.

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Cuticle Thickening in a Pyrethroid-Resistant Strain of the Common Bed Bug, Cimex lectularius L. (Hemiptera: Cimicidae)

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Abstract

Thickening of the integument as a mechanism of resistance to insecticides is a well recognised phenomenon in the insect world and, in recent times, has been found in insects exhibiting pyrethroid-resistance. Resistance to pyrethroid insecticides in the common bed bug,*Cimex lectularius* L., is widespread and has been frequently inferred as a reason for the pest's resurgence. Overexpression of cuticle depositing proteins has been demonstrated in pyrethroid-resistant bed bugs although, to date, no morphological analysis of the cuticle has been undertaken in order to confirm a phenotypic link. This paper describes examination of the cuticle thickness of a highly pyrethroid-resistant field strain collected in Sydney, Australia, in response to time-to-knockdown upon forced exposure to a pyrethroid insecticide. Mean cuticle thickness was positively correlated to time-to-knockdown, with significant differences observed between bugs knocked-down at 2 hours, 4 hours, and those still unaffected at 24 hours. Further analysis also demonstrated that the 24 hours survivors possessed a statistically significantly thicker cuticle when compared to a pyrethroid-susceptible strain of *C. lectularius*. This study demonstrates that cuticle thickening is present within a pyrethroid-resistant strain of*C. lectularius* and that, even within a stable resistant strain, cuticle thickness will vary according to time-to-knockdown upon exposure to an insecticide. This response should thus be considered in future studies on the cuticle of insecticide-resistant bed bugs and, potentially, other insects.

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Introduction

Since the turn of the century there has been a dramatic resurgence across many countries in the number of infestations of bed bugs, both the common, *Cimex lectularius* L., and tropical *Cimex hemipterus* F. species [1]. Insecticide resistance has been commonly inferred as a key driver of the bed bug resurgence [1–5] as pyrethroid resistance has been found to be concomitantly widespread with the growth and spread of infestations [4, 6–16]. Several mechanisms have been discovered that contribute to the observed resistance, including *kdr*-type target-site protein mutations [8, 9, 15, 16], overexpression of detoxifying enzymes [15,17], and reduced penetration [11, 18].

Reduced penetration in other insecticide-resistant insects has been known to be a resistance mechanism since it was first established in the 1960s with pyrethrin [19], organophosphates [20–22], carbamates [23, 24], and organochlorines [25–28]. It can occur via multiple mechanisms, including enhanced expression of metabolic resistance mechanisms in the integument [18], the increased presence of binding proteins, lipids, and/or sclerotization that trap insecticides [29, 30], a measurably thicker cuticle [31, 32], or a combination of some or all of these mechanisms together [30, 33–35]. Several of these mechanisms have been found in bed bugs with, to date, the exception of cuticle thickening.

Ordinarily, reduced penetration does not, by itself, impart a high degree of resistance [25, 33,36], although it may nonetheless have importance by way of conferring a level of cross-resistance to a wider variety of insecticides [25, 33, 37], increasing the efficiency of metabolic detoxification [26, 38–40], or delaying the onset of knockdown [25, 32, 41]. However, the expression of one or more resistance mechanisms does not necessarily predicate a corresponding change in expression of cuticular proteins [42] although it appears that, conversely, reduced penetration is typically found only when other mechanisms are present [32, 43–45].

No measurable comparison of the cuticle thickness has thus far been undertaken in bed bugs, although evidence from very recent molecular analysis of several *C. lectularius* strains point toward changes in protein expression [11, 39]. The manifestation and intensification of other resistance mechanisms in the cuticle is strongly indicative of reduced penetration and/or cuticle thickening [18] and this has most recently been successfully established in the South African malaria vector mosquito *Anopheles funestus* [32]. Thus, in adapting the experimental design used with *Anopheles funestus*, it is the aim of this paper to investigate the potential presence of cuticle thickening in a highly pyrethroid-resistant strain of *C. lectularius* and to examine the relationship of any such thickening to time-to-knockdown upon exposure to an insecticide.

Experimental Methods

Chemicals

Demand Insecticide® (25 g/L lambda-cyhalothrin) was supplied free of charge by Syngenta Crop Protection Pty Limited (Level 1, 2–4 Lyonpark Road, Macquarie Park NSW 2113).

Bed bug strains

Storage and culturing of the bed bug strains were conducted as approved by the Westmead Hospital Animal Ethics Committee (WHAEC Protocol No.2002) and in accordance with NSW Animal Research Review Panel (ARRP) Guidelines for the Housing of Rats in Scientific Institutions.

Cimex lectularius specimens were collected from a single, domestic, field infestation in the suburb of Parramatta, New South Wales, Australia, in December 2012. A detailed treatment history was not available for this strain, although it is believed insecticide control had been attempted prior to its collection. The specimens were examined for species, [46] *kdr* haplotype [8] and, thereafter, used to establish a colony in the departmental insectary maintained at 25°C (±1°C) and 75% (±10%) RH with a photoperiod of 12:12 (L:D) h [12]. All bed bugs were offered a blood meal on specific-pathogen-free (SPF) anaesthetised rats seven days prior to being selected for use in bioassays. Rat anaesthesia was achieved with an intraperitoneal injection of ketamine and xylazine, and recovery monitored until complete. No insecticide selection was undertaken. Resistance profiling indicated this strain homogeneously possessed haplotype B (L925I only) *kdr*-type resistance [8]. Exposure to a d-allethrin based rapid resistance field assay also failed to elicit any statistically significant levels of knockdown [47]. Given the lack of efficacy with d-allethrin (a first generation, type I pyrethroid), a second-generation type II pyrethroid, lambda-cyhalothrin, was preferentially selected for use as the screening agent in this study.

An additional laboratory strain of *C. lectularius* kept in colonies since the 1960s with known susceptibility to pyrethroids (designated the 'Monheim' strain [12]) was also used selectively to assess for any effect due to variations in insect body size. The Monheim strain lacks any *kdr*-type mutation (haplotype A) [8].

To ensure all bugs used in the experiments were of equal age, cohorts of fifth instar bugs were isolated from the stock colonies, fed to repletion, and monitored daily for appearance as adults. Once matured the bugs were immediately separated by sex to limit the potential for breeding, and aged for a further 8 or 9 days (depending on their intended use) without any further blood meal.

Knockdown bioassay

A 20 mL/L solution of Demand Insecticide was mixed according to label directions and applied at the rate of 1.2 mL (to the point of run-off) to 90 mm Ø Whatman™ No. 1 qualitative filter papers (Cat. No. 1001–090) held in petri dishes. Sixteen replicates of 10 male Parramatta strain

9-day-old bed bugs were then immediately placed onto the treated surface (no drying period) and knockdown was recorded at 10-minute intervals up to 4 hours. Knockdown was defined as the bugs not being able to right themselves when inverted onto their dorsal side. Affected bugs were immediately transferred to labelled specimen vials containing 70% ethanol. Bed bugs that were knocked-down within the first 2 hours were marked as the 'intolerant' group, and unaffected bugs at 4 hours were marked as the 'tolerant' group. The experiment was repeated until at least 10 bugs were collected for each response group.

In order to control for the additional time required in selecting for the most resistant bed bugs, the above experiment was repeated with a cohort of 8-day-old Parramatta strain male bugs, and the experiment (9 replicates of 10 bugs) allowed to run for 24 hours of continuous forced exposure. Any bed bugs unaffected at the conclusion of 24 hours were then marked as the 'resistant' group, and were thus 9 days old at the point of sampling as per the 'intolerant' and 'tolerant' response groups.

Exposure of the Monheim strain bugs to the above regimen was attempted, however, all bugs rapidly succumbed to the insecticide within a single 10-minute period and thus no separation into groups of varying tolerance was possible. Consequently, the Monheim strain was excluded from the inter-group cuticle thickness and time-to-knockdown analysis, but a random sample of 9-day-old bugs were nonetheless processed for cuticle measurements in order to provide for a comparison between 'susceptible' and 'resistant' bugs.

Preparation for scanning electron microscope

Specimens preserved in 70% ethanol were examined under the dissecting microscope in fresh 70% ethanol. The middle legs of each specimen were dissected from the body at the apical region of the femur. A subsequent severance was made at the tibia midpoint whilst the legs were suspended in a small drop of 70% ethanol. The remaining specimen bodies were stored in 70% ethanol for later analysis. Legs were moved to Eppendorf tubes, washed twice more in 70% ethanol to remove sectioning debris and processed in an ascending ethanol series as follows: 80%, 90%, 95% and 100%, twice for 5 mins each, followed by 3 washes for 10 minutes each in ultrapure ethanol. Samples were chemically dried for 3 mins in hexamethyldisilazane (Proscitech), transferred to new Eppendorf tubes and air-dried for 5 mins. SEM specimen stubs (Ted Pella) were modified with a diamond saw to expose a flattened surface. Legs were mounted to this surface with carbon tape and carefully positioned to ensure the tibia was vertically positioned and the severed midpoint was exposed at the stubs apical surface. Carbon paint was applied to secure samples to the specimen stubs. Specimens were sputter coated with gold for 2 mins at 25 mA with the EMITECH K550X and were stored in a desiccator.

Electron microscope imaging

Secondary electron micrographs were obtained with a Zeiss EVO 50 SEM at 10 kV. Each sample was individually rotated and tilted towards the secondary electron detector, varying the working distance between 9 and 25 mm. Image focus and scale calibrations were corrected with the dynamic focus and tilt correction tools. Integrated line averaging was utilised to minimise charging artefacts during image acquisition.

Image analysis

Raw and processed images were analysed in ImageJ (version 1.46r) [48]. To assess cuticle width, the known scale in microns from the processed imaged was measured according to the number of pixels, and this value transferred to the raw image. Thereafter, 12 point-to-point measurements were made, roughly equidistantly, from the outermost to innermost visible sections of the cuticle, whilst avoiding obvious debris, damage, or setae beds (Fig 1). Measurement of the various specimens was made while blinded to the allocated response group ('intolerant', 'tolerant', 'resistant' and 'susceptible') in order to limit the potential for bias.

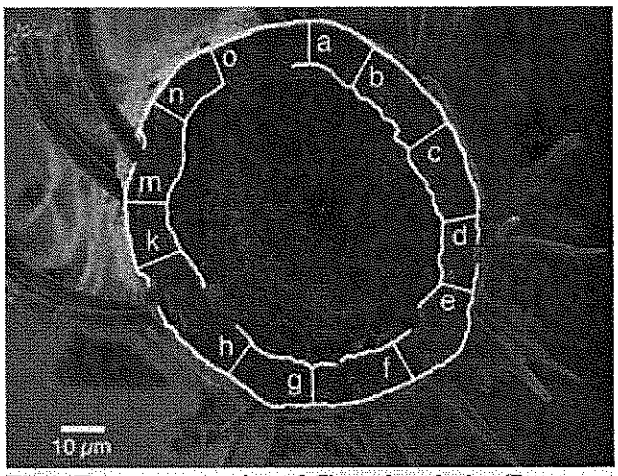

Fig 1. Transverse section of *Cimex lectularius* (Parramatta strain) middle leg tibia and example of twelve point-to-point cuticle measurements methodology.

Letters denote measurements of: a = 9.15 µm, b = 9.82 µm, c = 10.25 µm, d = 7.91 µm, e = 7.16 µm, f = 8.42 µm, g = 8.02 µm, h = 8.41 µm, k = 10.12 µm, m = 8.82 µm, n = 8.31 µm, o = 8.09 µm [i, j and l not included for purpose of legibility].

Sample sizes were balanced by random selection in IBM SPSS Statistic v22 for Mac (IBM Corp., 2013) with mean cuticle thickness values of the three Parramatta response groups compared via one-way ANOVA and Fisher's Least Significant Difference (LSD) post-hoc tests. Cuticle thickness vs. time-to-knockdown was analysed by linear regression. Cuticle thickness between 'susceptible' and 'resistant' bugs was compared via a one-way ANOVA.

Insect body measurements

Additional body measurements were made of the specimens from all four response groups used for cuticle analysis in order to check for any effect of varying body or appendage size on the resultant mean cuticle values. Several target measurements were adapted from previously published research [49], and included the pronotum width (*pw*), pronotum length (*pl*), head width (*hw*), length of the 1st antennal segment (after the scape–*a2l*), hind leg tibia length (*t3l*), and hind leg tibia width (*t3w*). Measurements were made under dissecting microscope using stage and/or optical micrometers and evaluated for statistical significance and effect sizes (Partial Eta^2 values) using general linear model multivariate analysis and Bonferroni post-hoc comparisons.

Results

Knockdown

Knockdown of the exposed 9-day-old Parramatta-strain bed bugs occurred asymptotically up to a value of approximately 76% (S.E. ± 1.8%) over the course of 4 hours, with the first bugs affected after 50 minutes (Fig 2) and increasing up to 38% (S.E. ± 6.5%) after 2 hours (the arbitrary end-point of 'intolerant' response group). Bed bugs aged to 8 days and continually force-exposed for 24 hours (to an equivalent age of 9-days-old) had knockdown of 82% (S.E. ± 2.0%), leaving a further 18% of bugs that were unaffected and thereafter classed as the 'resistant' group.

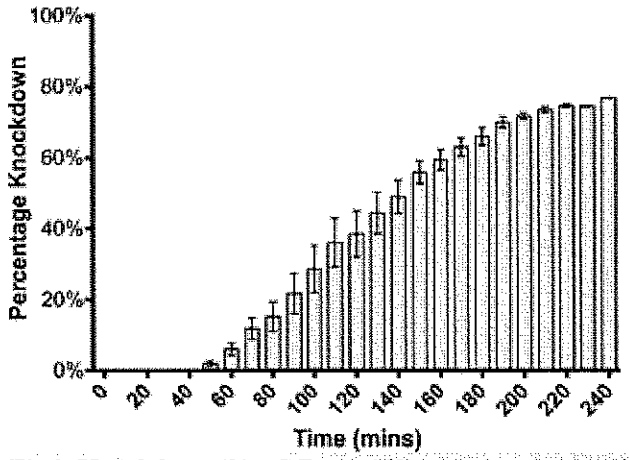

Fig 2. Knockdown (% ± S.E, n = 160) over time (minutes) of Parramatta strain *Cimex lectularius* upon forced exposure to wet residues of 20 mL/L Demand Insecticide® (25 g/L lambda-cyhalothrin).

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Imaging and cuticle thickness

Several specimens had to be excluded from analysis at the imaging stage due primarily to damage that had occurred during sectioning and mounting, or as a result of internal tissues obscuring the cuticle. Where this affected overall parity of sample size, excess specimens were excluded via random sampling in SPSS such that, in total, measurements from 10 specimens were eventually analysed from each response group.

Mean cuticle thickness was found to be significantly different ($p < 0.001$) between the three Parramatta-strain response groups (Fig 3). 'Resistant' bed bugs had a mean cuticle thickness of 10.13 μm (S.E. ± 0.15 μm), 'tolerant' bugs 9.51 μm (S.E. ± 0.26 μm) and 'intolerant' bugs 8.73 μm (S.E. ± 0.18 μm). 'Resistant' bugs thus had a significantly different mean cuticle thickness of +0.62 μm and +1.4 μm compared to the 'tolerant' ($p < 0.38$) and 'intolerant' ($p < 0.001$) bugs respectively, and the 'tolerant' bugs were also found to have a significantly different mean cuticle thickness of +0.78 μm compared to the 'intolerant' bugs ($p < 0.012$). Linear regression analysis of the time-to-knockdown and mean cuticle thickness (Fig 4) revealed a significant

trend ($p < 0.01$), with cuticle thickness positively correlated (R^2 adjusted = 0.337) with increasing time-to-knockdown. Given the relatively limited sample sizes and length between time intervals the otherwise modest adjusted R^2 value is, in context, a valid result.

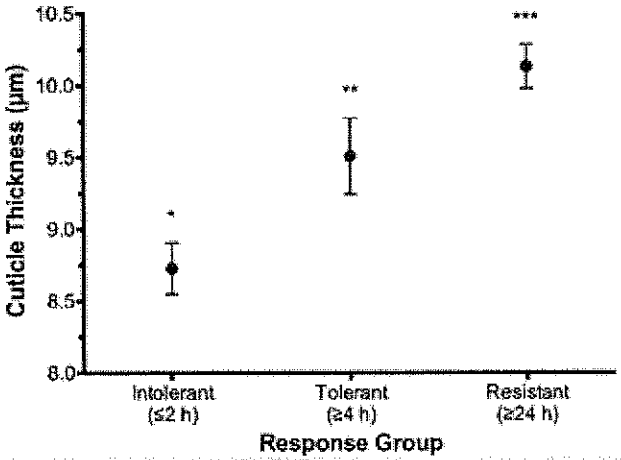

Fig 3. Mean cuticle thickness (µm ± S.E) of intolerant (n = 10), tolerant (n = 10) and resistant (n = 10) response group Parramatta strain *Cimex lectularius*.
Asterisks (*) indicate statistical significance ($p < 0.05$).

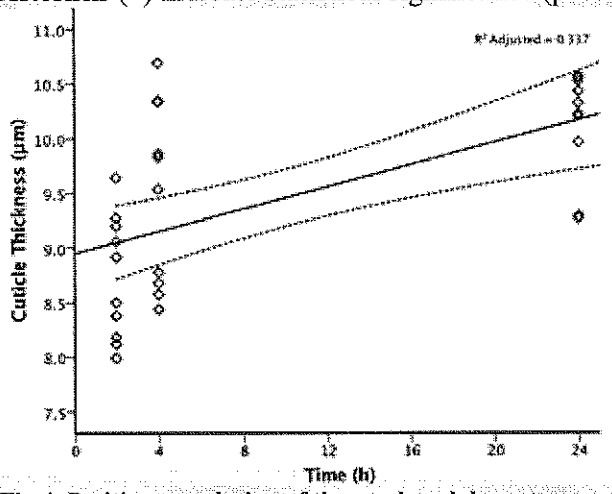

Fig 4. Positive correlation of time-to-knockdown to mean cuticle thickness of Parramatta strain *Cimex lectularius* (n = 10 per response group) upon continuous forced-exposure to wet residues of 20 mL/L Demand Insecticide® (25 g/L lambda-cyhalothrin).

Insect measurements

No statistically significant differences existed within the Parramatta strain between the three response groups for all the various body measurements (pronotum width, pronotum length, head width, length of the 1st antennal segment, hind leg tibia length, hind leg tibia width and ratio of tibia width to cuticle thickness), except cuticle thickness as noted separately above.

Monheim strain pyrethroid-susceptible bugs were found to be statistically significantly larger than the Parramatta strain bugs in pronotum width (all groups $p < 0.001$), pronotum length ('intolerant' $p < 0.002$; 'tolerant' $p = 0.042$), head width (all groups $p < 0.001$), antennal length

(all groups $p < 0.002$), and tibia length (all groups $p < 0.002$). No statistical significant differences were observed for tibia width (all groups $p > 0.05$), and pronotum length ('resistant' $p > 0.05$).

Previously, head width in *C. lectularius* has been established as correlated both to differences in the size of specimens between strains and differences in body extremities relative to overall body size [49]. In this study, examination of Partial Eta^2 (corrected) values similarly indicated that both pronotum width (0.730) and head width (0.759) were also prominent factors in accounting for the proportion of variance associated with each of the aspects measured. Consequently, head width was selected as the most appropriate feature to correct for size differences between the Monheim and Parramatta bed bug strains in order to make an appropriate comparison of mean cuticle thickness. To this effect, the observed difference in mean head width between the Monheim strain and Parramatta 'resistant' group bugs was found to be +9.9%. When this strain size difference is adjusted and factored into the cuticle thickness comparison, 'resistant' bugs had a significantly different ($p < 0.001$) mean cuticle thickness of +1.35 μm compared to the 'susceptible' bugs (Fig 5).

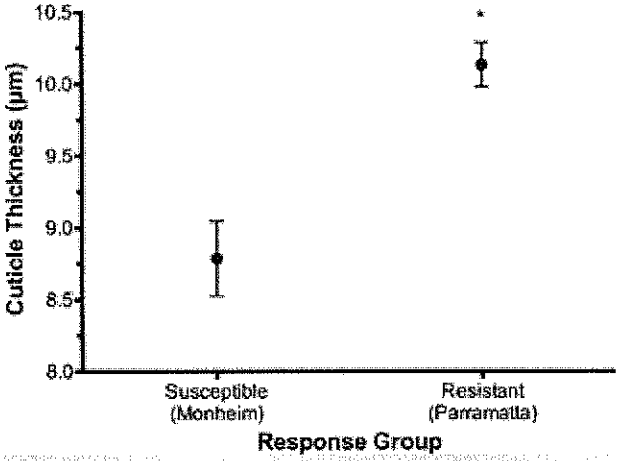

Fig 5. Mean cuticle thickness (μm ± S.E) of Monheim susceptible (n = 10) and Parramatta resistant (n = 10) *Cimex lectularius*.
Asterisks (*) indicate statistical significance ($p < 0.05$).

Discussion

Insecticide resistance is a genetic change in response to selection by toxicants that impairs control in the field [50] and is considered to be a natural evolutionary response to human-induced environmental stress [33, 51–53]. In the case of bed bugs, this would be failure of pest management attempts at infestation eradication through insecticidal means. Development or enhancement of the cuticle as the primary barrier to insecticides is integral to this process with an obvious advantage (in the absence of any other fitness costs) conferred to the bed bug phenotype of higher tolerance to forced insecticide exposure. Using a well-established time-to-knockdown method enables identification and separation of bugs of varying insecticide tolerance. In this study, the most resistant bed bugs (themselves separated from a highly resistant field strain) were found to have cuticle thickness 16% greater than the least resistant ('intolerant') bugs and 6.5% from the intermediately resistant ('tolerant') bugs. Further analysis (after correcting for strain

size differences) indicated the 'resistant' bugs possessed cuticle thickness 15.3% greater than that of a susceptible strain, thereby confirming a link between increased cuticle thickness and observed insecticide resistance.

Under normal field circumstance it would be expected that some variation in cuticle phenotype within a strain could be apportioned to factors such as diet or age. However, both diet and age were controlled prior to forced-selection of the bugs based on research that has shown bed bugs are appreciably resistant and express a stable metabolism at 9 days [54, 55], and that no difference in efficacy has been observed regardless of whether bugs have been fed previously (e.g. at day 1) or starved for up to 9 days [56]. This aligns with the practices of other laboratories employing bed bugs in insecticide-based bioassays, with most selectively using male bed bugs, aging between 7–10 days after emergence, and not feeding for 5–8 days prior to the experiment(s), if at all [6, 11, 13, 57–59].

A difference in cuticle thickness between strains of varying insecticide susceptibility was established in this study, after corrections were made for the increased size of the susceptible strain over the resistant strain. Given the Monheim strain bed bugs were found to be larger than the Parramatta strain bugs across a range of physical features (pronotum width, pronotum length, head width, antennal length, and tibia length) it is evident that overall insect size is a factor that should be considered in any future studies targeting morphological features if more than one field strain is being examined. Use of morphological features, and particularly measurement of head width, has been used to analyse evolutionary aspects of bed bugs [49] and our results suggest a similar approach may be warranted when examining other physical mechanisms that may confer reduced insecticide susceptibility in *C. lectularius*.

Thickening of the cuticle in resistant bed bugs may have important consequences for field control as the delivery of insecticides becomes potentially less efficient. However, further study of the cuticle layers, the relative contribution of each to the overall thickness, and the process of cuticle development and deposition in resistant bed bugs may be required in order to understand to what degree the cuticle thickening is reducing penetration (either alone or by enhancement with other resistance mechanisms). This, in turn, may influence advancements in insecticides and formulations in an effort to overcome the increased tolerance of bed bugs. Ideally, this research should be repeated with more field strains of both the common and tropical species of bed bug, however, the high cost of SEM research (currently USD$5000 per strain) was a limiting factor in this study. Nonetheless, further work is ongoing on a full examination of other resistance mechanisms within this and other field strains from Australia.

Conclusions

This study demonstrates that the most highly resistant bed bugs from a pyrethroid-resistant field strain possess a thicker cuticle that enables those bugs to survive exposure to insecticides when compared both to less tolerant insects from the same strain and also those from a susceptible strain. This may help to explain why failures in the control of field infestations are so common, and further emphasizes the need for an integrated approach in the control of bed bugs to prevent the further spread of highly resistant insects. Changes in cuticle thickness, the lipid composition and passage of insecticides, if combined with expression of detoxifying enzymes or target-site

insensitivity, may also have important consequences for the use of formulated insecticides for the field control of bed bugs.

Acknowledgments

Storage and culturing of the bed bug strains were conducted as approved by the Westmead Hospital Animal Ethics Committee (WHAEC Protocol No.2002) and in accordance with NSW Animal Research Review Panel (ARRP) *Guidelines for the Housing of Rats in Scientific Institutions*. The authors wish to thank the staff of Westmead Hospital Department of Animal Care and the Department of Medical Entomology for their assistance with colony maintenance. The authors acknowledge the facilities and the scientific and technical assistance provided by Naveena Gokoolparsadh and Jenny Whiting of the Australian Microscopy & Microanalysis Research Facility at the Australian Centre for Microscopy & Microanalysis at the University of Sydney.

Author Contributions

Conceived and designed the experiments: DGL SLL CEW SLD. Performed the experiments: DGL SLL. Analyzed the data: DGL. Contributed reagents/materials/analysis tools: DGL SLL. Wrote the paper: DGL SLL CEW SLD.

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Dynamics of bed bug infestations and control under disclosure policies

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Bed bugs have reemerged in the United States and worldwide over recent decades, presenting a major challenge to both public health practitioners and housing authorities. A number of municipalities have proposed or initiated policies to stem the bed bug epidemic, but little guidance is available to evaluate them. One contentious policy is disclosure, whereby landlords are obligated to notify potential tenants of current or prior bed bug infestations. Aimed to protect tenants from leasing an infested rental unit, disclosure also creates a kind of quarantine, partially and temporarily removing infested units from the market. Here, we develop a mathematical model for the spread of bed bugs in a generalized rental market, calibrate it to parameters of bed bug dispersion and housing turnover, and use it to evaluate the costs and benefits of disclosure policies to landlords. We find disclosure to be an effective control policy to curb infestation prevalence. Over the short term (within 5 years), disclosure policies result in modest increases in cost to landlords, while over the long term, reductions of infestation prevalence lead, on average, to savings. These results are insensitive to different assumptions regarding the prevalence of infestation, rate of introduction of bed bugs from other municipalities, and the strength of the quarantine effect created by disclosure. Beyond its application to bed bugs, our model offers a framework to evaluate policies to curtail the spread of household pests and is appropriate for systems in which spillover effects result in highly nonlinear cost–benefit relationships.

bed bugs | disclosure | mathematical model

Bed bugs (*Cimex lectularius*) have reemerged in the United States and worldwide since the early 2000s (1–4). The prevalence of infestations in major US cities is high, although only poorly described. In 2014, the New York City Community Health Survey estimated the annual prevalence of bed bug infestations to be 5.1% city-wide and as high as 12% in some neighborhoods (5). Similarly, a door-to-door survey of bed bug infestations conducted in a Philadelphia census tract in 2013 found that 11.1% of respondents had recent bed bug infestations (6).

The health consequences of the current bed bug pandemic are inarguably enormous. Bed bugs inflict physical and psychological distress to those they bite and whose dwellings they infest, causing itching, rashes, allergies, sleep loss, anxiety, and other symptoms (7–9). In addition to these direct effects, bed bug infestations prevent homebound patients—especially senior citizens and disabled individuals—from receiving care, as many home-care providers are reluctant to enter infested houses (10, 11). Poisoning by insecticides inappropriately applied to combat bed bugs has caused at least one fatality and left scores acutely ill and countless more exposed, often unknowingly (12). Bed bugs are competent vectors of *Trypanosoma cruzi* (13, 14) and *Bartonella quintana* (15), the etiological agents of Chagas disease and trench fever, respectively. Whether or not bed bugs currently are, or will become, epidemiologically relevant in the transmission of infectious agents remains unknown.

The optimal political response to the bed bug epidemic has yet to be determined. Policies must balance the rights of tenants, landlords, and the public at large. Treatment of infestations with insecticide or heat-based interventions is generally paid for by individuals, not municipalities, and so policies strive to incentivize rapid and effective treatment while minimizing stigma, cost, and lost housing opportunities. An increasing number of US states and municipalities are responding to the rise in bed bug prevalence with disclosure policies, which require landlords to notify potential tenants of bed bug infestation histories. For example, in New York City, landlords are required to disclose the bed bug infestation and treatment histories of their units for the previous year to all tenants entering a lease agreement (16). Similar, though less stringent, versions of this policy have been passed in San Francisco (17); Mason City, Iowa (18); Connecticut (19); and Maine (20).

The primary aim of disclosure policies is to protect individuals from unknowingly leasing an infested rental unit. Nonetheless, these policies may have community-wide effects: Disclosure can decrease the desirability of infested units, thereby imposing a kind of partial quarantine that could decrease the prevalence of infestations on a city or even regional scale. The potential benefits of disclosure seemingly come at a cost,

Significance

Bed bugs are household pests that bite humans and cause myriad medical, psychological, social, and economic problems. Infestation levels have resurged across the United States in recent decades, and cities and states are debating strategies to deal with them. Here, we introduce a mathematical model to study the spread of bed bugs and predict the costs and benefits of policies aimed at controlling them. In particular, we evaluate disclosure, a policy that requires landlords to notify potential tenants of recent infestations in a unit. While disclosure aims to protect individual tenants, our results suggest that these policies also reduce infestation prevalence market-wide. Disclosure results in some initial cost to landlords but leads to significant savings in the long term.

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Data deposition: All analyses and figures presented in *Results* and *SI Appendix* can be reproduced using code available at https://github.com/sherriexie/bedbugdisclosure. An R Shiny web application allowing users to simulate the authors' model and additional animation are available at https://bedbugdisclosure.shinyapps.io/shinyapp/.

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most of which would fall on the shoulders of landlords. These costs could manifest as both direct costs of pest-control treatment and lost rent due to increased vacancies and tenant turnover under disclosure. Disclosure laws have been actively contested by landlord organizations (21), and opponents fear they could stigmatize affected buildings and lower property values (22). But the infectious nature of bed bug infestations creates spillover effects that result in highly nonlinear cost–benefit relationships. If bed bug prevalence decreases following more proactive disclosure legislation, fewer treatments and less tenant turnover could increase the value of rental properties.

Here, we introduce a simple mathematical model to estimate the financial impact of bed bug disclosure policies on landlords over a range of time horizons. Our model adapts the traditional Susceptible–Infectious–Susceptible (SIS) framework originally developed to model infectious diseases (23) to capture housing-market dynamics. To understand the general behavior of this system, we derive the basic reproductive ratio, R_0, which provides insight on whether, over the long term, the prevalence of infestation is likely to trend toward zero or reach an endemic equilibrium. We use parameters derived from field studies and publicly available sources that are meant to approximately represent a single rent level in a major US city. In developing our model, we describe a shift from costs to savings associated with disclosure over a relatively short time horizon. In subsequent sensitivity analyses, we identify the key parameters driving our results.

Methods

A Susceptible-Infested-Susceptible Model for Bed Bug Transmission. Our model of bed bug transmission is an adaptation of the traditional SIS model (23). Here, the unit of infection is a rental unit, which we define to be any apartment unit, condominium, or other single-family dwelling that is rented or available for rent. Units free of bed bugs are considered susceptible (S), whereas infested (I) units harbor bed bugs. Due to the resilience of bed bug populations, we assume that a unit can move out of the I class only by receiving treatment. We consider a closed population of rental units of a constant size, N. In the basic model (Fig. 1), the S and I classes are further subclassified as renter-occupied (r) or vacant (v) depending on tenant occupancy, yielding a total of four classes: susceptible-occupied (S_r), infested-occupied (I_r), susceptible-vacant (S_v), and infested-vacant (I_v). The system of differential equations of the SIS model is as follows:

$$\begin{aligned}
\frac{dS_r}{dt} &= -\beta S_r I_r/N + \gamma I_r + n(1 - kf(t))S_v - mS_r \\
\frac{dI_r}{dt} &= \beta S_r I_r/N + kf(t)nS_v + nI_v - (\gamma + bm)I_r \\
\frac{dS_v}{dt} &= mS_r + \gamma I_v - nS_v \\
\frac{dI_v}{dt} &= bmI_r - (\gamma + n)I_v \\
N &= S_r + I_r + S_v + I_v \\
f(t) &= \frac{bI_r}{S_r + bI_r}.
\end{aligned} \qquad [1]$$

Individuals move out of occupied units at a baseline rate m, therefore spending an average time $1/m$ in a given unit before moving. We assume that a bed bug infestation in an occupied unit increases the move-out rate by a factor b ($b > 1$). New tenants move into vacant units (whether they are susceptible or infested) at a rate n, so that $1/n$ is the average time a unit remains vacant. Infested units are treated at rate γ, which implies that the average time for an infested unit to successfully initiate and complete treatment is $1/\gamma$. Units can become newly infested by two mechanisms in our model: infectious transmission and relocation transmission. Both of these modes of transmission contribute to the system's basic reproductive ratio, R_0, presented in *Results*. In infectious transmission, susceptible-occupied units become infested through importation of bugs on clothes, furniture, etc. at a rate assumed to be proportional to the infectivity, β, and the total prevalence of infested-occupied units in the population (i.e., I_r/N). In relocation transmission, individuals who have moved out of an infested unit may inadvertently bring bed bugs to the next unit they rent and seed a new infestation. We assume that the fraction of susceptible-vacant units that become infested via relocation transmission is the product of the presumed fraction of new occupants coming from previously infested apartments ($f(t)$) and the probability that these occupants establish bed bug populations in their new units upon arrival (k). $f(t)$ is the ratio of the movement out of infested units (bmI_r) and the movement out of all rented units ($mS_r + bmI_r$), or $f(t) = bI_r/(S_r + bI_r)$. Model parameters are listed and summarized in Table 1.

We make a few simplifying assumptions in assembling this model. We assume that rental units are homogeneous with respect to their market parameters (move-in and -out rates) and their bed bug transmission parameters (susceptibility to bed bugs, treatment rate, etc.). We also adopt the homogeneous mixing assumption held by many infectious disease models (23). Applied to the context of housing, this assumption means that any

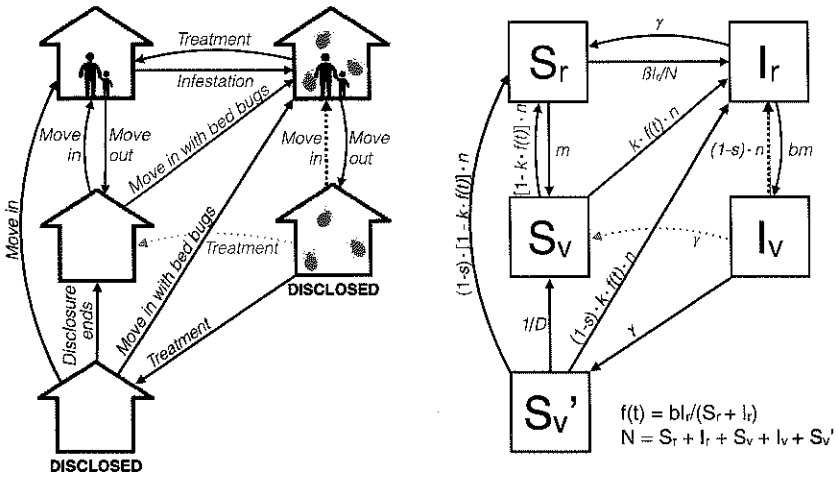

Fig. 1. A mathematical model of bed bug spread with and without disclosure policies. *Left* shows the processes leading to transfer between rental unit types, while *Right* lists the state variable names and transition rates. Items in black are in the model regardless of whether disclosure policies are implemented; items in red are only in the model with disclosure; and items in gray are only in the model without disclosure. Rental units are classified by both their occupancy status and infestation status: susceptible-occupied (S_r), infested-occupied (I_r), susceptible-vacant (S_v), and infested-vacant (I_v). When disclosure is implemented, there is an additional susceptible-vacant-disclosed class (S_v'), indicating a previous infestation which was treated. A detailed description of the transition rates is provided in the text. Parameter descriptions and values are in Table 1. The model equations are shown in Eqs. 1 and 2.

Table 1. Parameter point estimates and ranges used for sensitivity analysis

Parameters	Description	Best estimate	Range	Ref(s).
p (%)	Baseline prevalence	5	0.1–10	—
β	Infectivity	Fit	Fit	—
s	Renter selectivity	0.5	0.01–1	—
$1/\gamma$ (mo)	Average duration of infestation	6	2–12	—
k	Probability of relocation transmission	0.3	0–1	—
b	Vacancy multiplier	1.3	1–5	—
m (y^{-1})	Move-out rate	0.5	—	(24)
n (y^{-1})	Move-in rate	6	—	(24, 25)
D (y)	Length of disclosure	1	—	(16)
Cost				
Treatment (c_{trt}, \$)	Average cost of successfully exterminating a bed bug infestation	1,225	—	(1)
Vacancy (c_{vac}, \$)	Average cost of a rental unit lacking tenants for 1 mo	1,000	—	(26)
Turnover (c_{tov}, \$)	Average cost of turning over a rental unit to new tenants	1,000	—	(27)

two rental units have an equal probability of "interacting" with each other, via in-person visits, exchange of objects, or turnover of tenants. We discuss the implications of relaxing these assumptions in the final section of *Results*.

The Model in the Presence of Disclosure. We expand the model to account for the effects of disclosure policies (Fig. 1). Under this model, units in the I_v class are immediately disclosed and, upon treatment, move to a new susceptible-vacant-disclosed (S_v') class. The expanded model is as follows:

$$\begin{aligned}
\frac{dS_r}{dt} &= -\beta S_r I_r/N + \gamma I_r + n(1-kf(t))S_v + (1-s)n(1-kf(t))S_v' - mS_r\\
\frac{dI_r}{dt} &= \beta S_r I_r/N + nkf(t)S_v + (1-s)nkf(t)S_v' + (1-s)nI_v - (\gamma + bm)I_r\\
\frac{dS_v}{dt} &= mS_r + (1/D)S_v' - nS_v\\
\frac{dI_v}{dt} &= bmI_r - (\gamma + (1-s)n)I_v\\
\frac{dS_v'}{dt} &= \gamma I_v - ((1-s)n + 1/D)S_v'\\
N &= S_r + I_r + S_v + I_v + S_v'\\
f(t) &= \frac{bI_r}{S_r + bI_r}.
\end{aligned} \quad [2]$$

We assume all disclosed units are less desirable to potential tenants proportional to a renter-selectivity parameter (s). Units in the I_v and S_v' class might be thought to be in a "leaky quarantine," the strength of which is determined by s. When $s = 1$, no currently (I_v) or recently (S_v') infested units are rented out, meaning that disclosure results in a full quarantine. At the other extreme, when tenants do not change their renting behavior based on a unit's disclosure status ($s = 0$), the model reduces to the model in the absence of disclosure. We assume landlords comply fully with disclosure and units in class S_v' return to class S_v at rate $1/D$, where D represents the mandated disclosure period. Although in reality disclosure is of a fixed length, we have modeled it as a continuous transition for simplicity. In *SI Appendix*, we show that relaxing this assumption does not impact our conclusions.

Estimating Disclosure Costs. Our primary outcome of interest is the change in expected cost due to disclosure, which we define as the difference in cost to landlords in the presence of disclosure compared to the absence of disclosure. In the context of a rental market with bed bugs endemic, cost can take the following forms: bed bug treatment costs, rental turnover costs, and opportunity costs due to vacancy. Bed bug treatment costs are the expenses associated with the extermination or attempted extermination of bed bugs and include fees to pest-control companies and contractors. Turnover costs include the expenses involved in repairing, advertising, and showing units to prospective tenants. Opportunity costs due to vacancy are incurred anytime a rental unit lacks tenants and are equal to the rental price for that unit. Hereafter, we will use the term "cost" to refer to total additional cost to landlords that result from disclosure. Similarly, we will use the terms "treatment cost," "turnover cost," and "vacancy cost" to refer to these component costs with respect to disclosure (i.e., the difference of each component in the presence and absence of disclosure).

The average per-unit number of bed bug treatments occurring in a given year is equal to the number of transitions from infested to susceptible classes for that year divided by the number of rental units in the system N, and the average number of turnover events is equal to the number of transitions from vacant to occupied classes divided by N. Similarly, the average time that each unit is vacant is equal to the total unit-time spent in vacant classes divided by N. Then, if c_{trt}, c_{tov}, and c_{vac} are constants equal to the average ancillary cost of bed bug treatment, average cost of moving, and average cost of untreated infestation, respectively, the component costs of disclosure from the perspective of renters can be expressed for a given year Y by the following:

$$\begin{aligned}
\text{Treatment cost} &= \frac{c_{trt}}{N}\left(\int_Y^{(Y+1)} (\gamma I_r + \gamma I_v)dt\Big|_{s=s} - \int_Y^{(Y+1)} (\gamma I_r + \gamma I_v)dt\Big|_{s=0}\right)\\
\text{Turnover cost} &= \frac{c_{tov}}{N}\left(\int_Y^{(Y+1)} (nS_v + n(1-s)S_v' + n(1-d)I_v)\,dt\Big|_{s=s}\right.\\
&\quad \left. - \int_Y^{(Y+1)} (nS_v + n(1-s)S_v' + n(1-d)I_v)\,dt\Big|_{s=0}\right)\\
\text{Vacancy cost} &= 12\frac{c_{vac}}{N}\left(\int_Y^{(Y+1)} (S_v + S_v' + I_v)\,dt\Big|_{s=s}\right.\\
&\quad \left. - \int_Y^{(Y+1)} (S_v + S_v' + I_v)\,dt\Big|_{s=0}\right).
\end{aligned} \quad [3]$$

Above, each component cost is calculated as the difference in the average quantity of treatment, turnover, and vacancy in the presence vs. in the absence of disclosure multiplied by the cost constant. Cost can then be calculated as the sum of the treatment, turnover, and vacancy costs.

Model Implementation and Parameter Estimates. We ran the model with a total (N) of 1,000 units (although our results are insensitive to population size). The average cost of bed bug treatment was set to equal \$1,225, the median cost of bed bug treatment for single-family homes reported by a national survey of pest-management professionals in 2015 (1). The average cost of turnover was set equal to \$1,000, a figure that has been cited on property-management blogs (24). The average monthly rent (and monthly opportunity cost due to vacancy) was set to equal \$1,000, roughly the national median reported by the American Community Survey for 2017 (25).

Initial conditions for the start of each simulation were the equilibrium values for the same system in the absence of disclosure, assuming that the overall baseline prevalence of infestation, $(I_r + I_v)/N$, was p:

$$\begin{aligned}
S_r^* &= \frac{N}{m+n}\left((1-p)n - pm\frac{b\gamma}{bm+\gamma+n}\right)\\
I_r^* &= Np\frac{\gamma+n}{bm+\gamma+n}\\
S_v^* &= N\frac{m}{m+n}\left((1-p) + p\frac{b\gamma}{bm+\gamma+n}\right)\\
I_v^* &= Np\frac{bm}{bm+\gamma+n}.
\end{aligned} \quad [4]$$

Estimated values or ranges for parameters are reported in Table 1. Move-in and -out rates were estimated according to data from the US Census Bureau Housing Vacancy Survey for 2017 and the 2017 National Apartment Association Survey of Operating Income and Expenses in Rental Apartment Communities (26, 27). The move-out rate, m, was estimated based on the average frequency of moves (once every 2 y). To estimate the move-in rate (n), we calculated the percent of units that would be vacant in our model at baseline [percent vacant = $(S_v + I_v)/N$ in Eq. 4, $\approx m/(n+m)$ when prevalence is low] and chose a move-in rate so this matched the national average of 7% rental vacancy. The length of disclosure D was set to equal 1 y, which is equivalent to the length mandated by New York City (16). The infectivity β cannot be observed directly; we calculated it by solving for the value that would yield a given baseline prevalence p, which is more easily observed and interpreted:

$$\beta = \frac{N}{S_r^* I_r^*}\left((\gamma + bm)I_r^* - \frac{kbI_r^*}{S_r^* + bI_r^*}nS_v^* - nI_v^*\right), \quad [5]$$

where S_r^*, I_r^*, S_v^*, and I_v^* are given in Eq. 4.

Some parameters, including the average duration of infestation ($1/\gamma$), probability of relocation transmission (k), and vacancy multiplier (b), could not be estimated from available data; they were thus assigned realistic point values and assessed over ranges of values in subsequent sensitivity analyses. No data exist on the duration of bed bug infestations, although there are several factors impeding timely treatment. Recent genetic analyses suggest that infestations are founded by small populations consisting of few individuals or even a single mated female (28, 29), and reactions to bites are nonspecific and often misdiagnosed (30, 31), both of which retard detection by tenants and landlords. Even detection by pest-management professionals, which occurs by visual inspection and is sometimes aided by trained canines, has imperfect sensitivity and specificity (31). Moreover, treatment failure is common, even after multiple visits (32–34). Due to the challenges involved in bed bug detection and treatment, the average duration of infestation was estimated to equal 6 mo; sensitivity analysis evaluated how results changed if $1/\gamma$ were as brief as 2 mo or as long as 1 y.

There are anecdotal reports of tenants moving out of apartments prematurely due to bed bug infestations (35, 36), but data that can be used to estimate the factor by which infestation increases move-out rate are lacking. We chose a relatively conservative estimate for b (1.3, where move-out is assumed to be 30% greater in infested units relative to noninfested units) and found in subsequent sensitivity analyses that higher values of b led to even greater prevalence reduction and cost savings over the long term (*SI Appendix*, Fig. S9). Because bed bugs find harborage in furniture and clothing and much of their long-range dispersal is believed to be human-mediated (29), we reasoned that relocation transmission (whereby individuals moving out of an infested unit inadvertently bring bed bugs that seed a new infestation in their next unit) occurs, although at an unknown rate. Given the lack of data with which to estimate the probability of relocation transmission k, we set it to an intermediate value (0.3). Sensitivity analyses determined results to be robust to changes in k across its full range of possible values (*SI Appendix*, Figs. S6 and S7).

The model was coded and run in R using a differential equation solver in the *deSolve* library (37), and results were reported after each 1-y interval. An R Shiny web application allowing users to simulate our model themselves under alternate parameter values and visualize the output is available at https://bedbugdisclosure.shinyapps.io/shinyapp/. All analyses and figures presented in *Results* and *SI Appendix* can be reproduced by using code we have made available at https://github.com/sherriexie/bedbugdisclosure.

Results

Effects of Disclosure on Cost and Prevalence. Using our model and our best estimated parameter values (Table 1), we evaluated the impact of a newly implemented disclosure policy on the prevalence of bed bugs and the cost to landlords (Fig. 2). The cost of disclosure is high initially—reaching $25 per unit on the market after 2 y—but it decreases steadily, so that by year 5, landlords experience savings. The trends in total cost can be understood by examining the cost components. While turnover cost remains relatively constant and minimal, vacancy and treatment costs vary over time. Vacancy cost escalates directly after the implementation of disclosure, as disclosure makes infested and recently infested units less appealing to potential tenants. Meanwhile, this pseudo-"quarantine" of infested and recently infested units

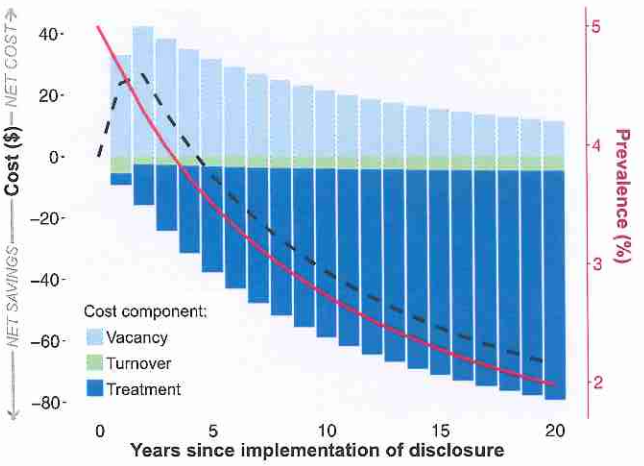

Fig. 2. Impact of disclosure on bed bug prevalence and cost over time. Cost to landlords, defined as the difference in average, per-unit cost in the presence of disclosure compared with no disclosure, is shown by the dashed black line. The components of cost are shown as bars representing averages over 1-y periods and are broken down into cost due to unrented vacant units ("vacancy"), cost due to treating infested units ("treatment"), and cost from moving tenants into vacant units ("turnover"). The overall prevalence of infestation in the population is shown by the solid, red line. The model was run by assuming that before the implementation of disclosure, the baseline prevalence of infestations was at a steady-state value of 5%. We assumed that disclosure discouraged but did not prevent rental of disclosed units ($s = 0.5$). Other parameter values are shown in Table 1, and results for additional parameter values are shown in *SI Appendix*.

causes a steady decrease in prevalence (Fig. 2). Accordingly, the cost of treatment starts slightly negative—reflecting a cost savings, and these savings increase over time as prevalence continues to decline. Because bed bug infestations increase vacancy due to the larger move-out rate ($b > 1$), the decline in prevalence also mitigates the effect of disclosure on vacancy; vacancy cost—although high initially—decreases over time. The net effect is that cost is high when disclosure is first introduced but quickly converts to savings that subsequently increase with time.

We examined in more detail how the predicted impact of disclosure policies depends on two parameter values that may vary between municipalities and are difficult to estimate: the baseline prevalence and the renter selectivity s (Figs. 3 and 4 and *SI Appendix*, Figs. S1 and S2). In all cases, year 5 marks the approximate turning point where vacancy costs are offset by savings from decreased treatment and total cost begins to dip below zero (Fig. 3*E*). In the initial years of disclosure, costs are greater if baseline prevalence is higher, and they are greatest when both the baseline prevalence and renter selectivity are high (Fig. 3 *A*–*D*). The initial effect of disclosure policies under parameter regimes where both baseline prevalence and renter selectivity are high is to increase vacant units and the associated vacancy costs. In later years, the trend between cost and baseline prevalence actually reverses, and higher baseline prevalence results in increased savings. The same combination of high baseline prevalence and high renter selectivity that resulted in the greatest cost during the initial years of disclosure results in the greatest savings in later years. If we discount costs and savings that occur in later years (using methods detailed in *SI Appendix*), the results are similar, although the eventual savings decrease by an amount that is commensurate with the discount rate (*SI Appendix*, Fig. S3).

The greater cost savings accrued in later years are mediated by the effect of disclosure on the overall bed bug infestation prevalence (Fig. 4). Reductions in prevalence are seen as long as tenants show any selectivity in favor of units with no disclosed

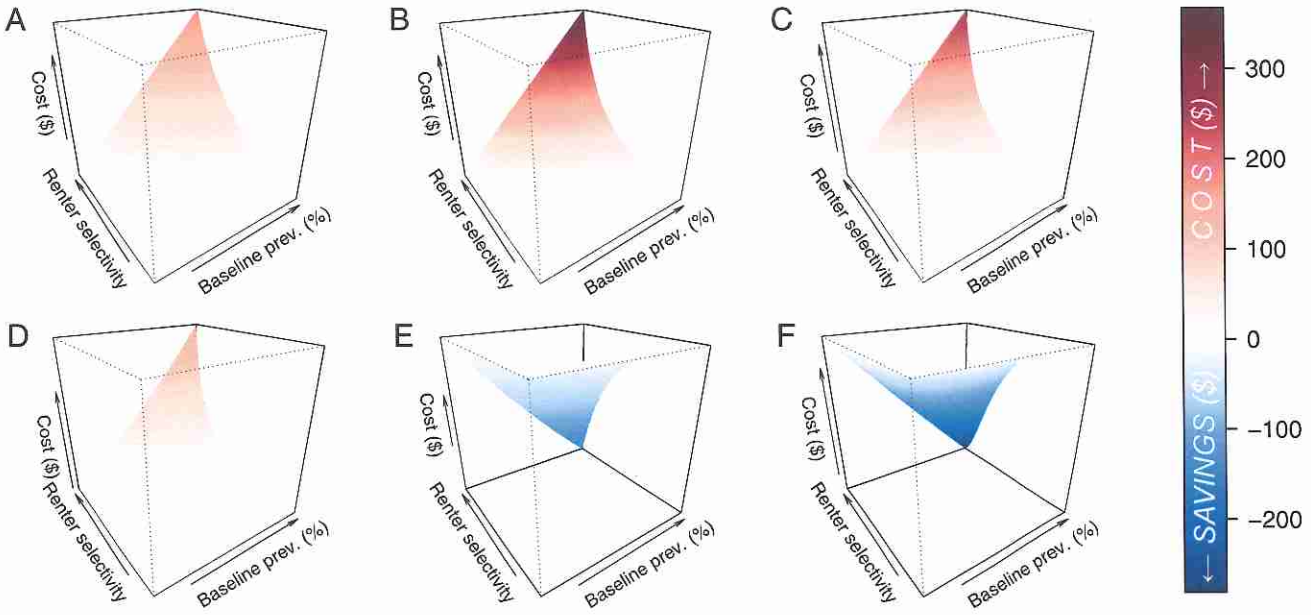

Fig. 3. Total per-unit cost due to disclosure over time, as a function of the baseline prevalence (prev.) and renter selectivity. Results are presented for years 1 (*A*), 2 (*B*), 3 (*C*), 4 (*D*), 5 (*E*), and 20 (*F*) after the implementation of a disclosure policy. Cost is calculated as the sum of the total cost in the population due to vacancy, treatment, and tenant turnover, for the given 1-y interval, averaged over the total rental units. Red indicates situations where the cost to landlords is higher due to disclosure, whereas blue indicates situations where costs have decreased from baseline (savings). Baseline prevalence (p) ranges from 0.1 to 10%, and renter selectivity (s) ranges from 0.01 to 1. To see the dependence of the cost components on these parameters, refer to *SI Appendix*, Fig. S1. An animation showing the dependence of cost on p and s over the initial 20 y of disclosure is available at https://bedbugdisclosure.shinyapps.io/shinyapp/.

bed bug history compared with units with a disclosed bed bug history ($s > 0$) but are more extreme for greater renter selectivity ($s \to 1$). This result holds at all times, and in some cases, we predict that prevalence can be driven to zero (bed bugs eliminated from the population). Overall, this analysis suggests that situations with unfavorable initial costs may be the same situations which lead to greater savings and prevalence reduction in the long run.

In addition to estimating disclosure costs for landlords, we also assessed the economic impact of disclosure on renters via methods outlined in *SI Appendix*. Disclosure policies are aimed at protecting tenants, and, as expected, we found this group to benefit financially from disclosure. Unlike landlords, who do not experience a net savings until later years, renters immediately benefit, with savings that grow over time as bed bug prevalence falls (*SI Appendix*, Fig. S4). In simulations with higher initial bed bug prevalence or higher renter selectivity (s), the savings in any given year are higher (*SI Appendix*, Fig. S5).

Analytic Results and Threshold Behavior. Similar to classic SIS infection models, our model of bed bug spread and control has two possible long-term outcomes: persistence of infection at an endemic equilibrium or decline of infestation levels toward zero. For any particular parameter set, one and only one of these outcomes represents a stable steady state of the dynamical system. We used the next-generation matrix method (38, 39) to calculate the basic reproductive ratio R_0 for our model as

$$R_0 = \left(\frac{n}{n+m}\beta + kbm\right)\left[\gamma + bm\frac{\gamma}{n(1-s)+\gamma}\right]^{-1}. \quad [6]$$

R_0 describes the average number of secondary infestations caused by the introduction of a single infested unit into a market of otherwise susceptible units. In addition, it determines the stability of the two possible equilibria for this model: Infestation persists as long as $R_0 > 1$ and declines to zero for $R_0 < 1$. In the absence of infestation (Eq. 4 with $p = 0$), a fraction $S_r^*(0)/N = n/(n+m)$ of units are rented, and $S_v^*(0)/N = m/(n+m)$ are vacant. The first part of R_0 (within the parentheses) describes the initial spread of infestations by two independent routes: infectious transmission of already rented units $[\beta S_r^*(0)]$ and relocation transmission of previously vacant units $[nkf(0)S_v^*(0) = kbm]$. The second part is the average time that an infested unit stays infested before being treated (either while still occupied or after being vacated). Disclosure reduces this infestation time by discouraging renters from moving into infested vacant apartments, preventing these units from contributing to interhousehold transmission.

For our baseline parameter values (Table 1 and β calibrated to give $p = 5\%$ steady-state prevalence), R_0 is near 1: In the absence of disclosure ($s = 0$), $R_0 = 1.05$, and it decreases to 0.88 as the renter selectivity increases to 1. R_0 is dominated by infectious transmission, while relocation transmission plays a more minor role (contributing ~8%). Note that the length of the disclosure period does not directly influence the value of R_0, and hence will not affect the persistence of infestation. Disclosure policies will have more impact on the value of R_0 when there is more apartment turnover (higher m and n) and when tenants are effectively turned off from disclosed rental units ($s \to 1$).

The functional form of R_0 also suggests that other additional policies could have a greater impact on reducing prevalence than disclosure alone. Policies that incentivize the rapid identification and treatment of bed bug infestations (increasing γ) are likely to have the greatest effect in terms of stemming the epidemic. Disclosure policies, if enacted well, may be among these, as landlords would likely wish to retain tenants in infested units, and treat these effectively, to avoid the requirement of disclosing infestations to future tenants.

Sensitivity of Results to Parameter Estimates. We analyzed the sensitivity of results to uncertainty in the estimates for the probability of relocation transmission (k), average duration of

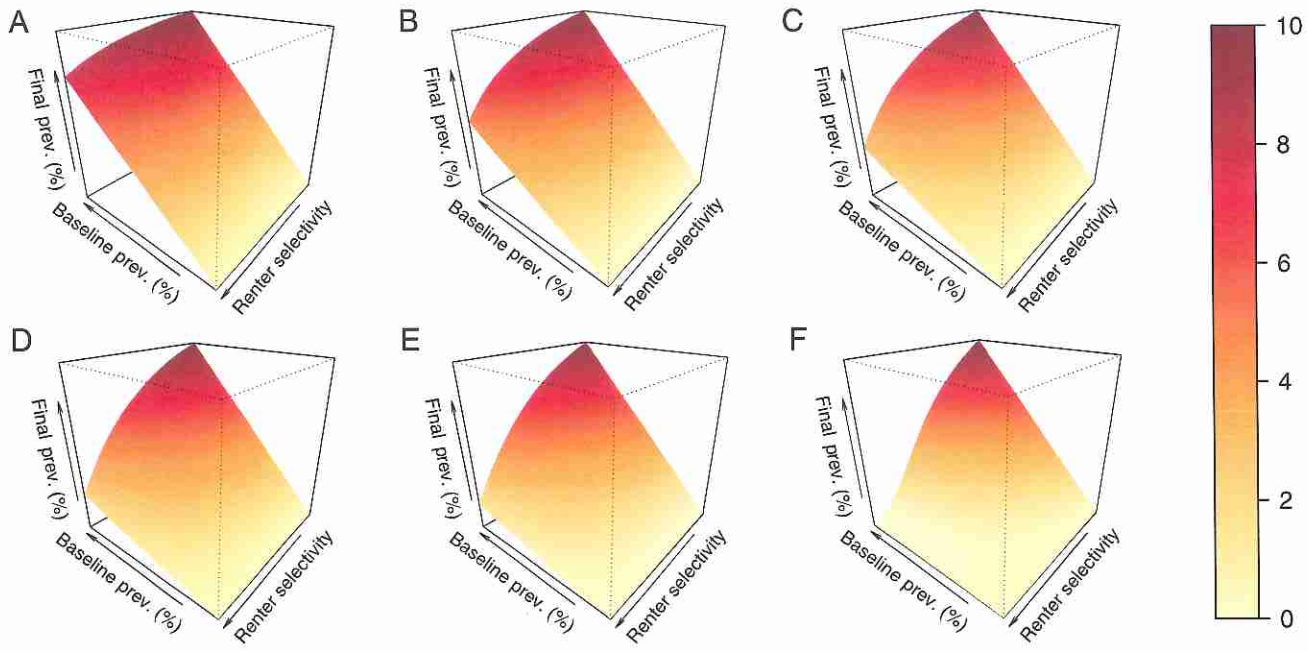

Fig. 4. Prevalence of bed bug infestations over time after implementation of disclosure, as a function of the baseline prevalence (prev.) and renter selectivity. Results are presented as the year-end ("final") prevalence for years 1 (*A*), 2 (*B*), 3 (*C*), 4 (*D*), 5 (*E*), and 20 (*F*) after the implementation of a disclosure policy. Baseline prevalence (p) ranges from 0.1 to 10%, and renter selectivity (s) ranges from 0.01 to 1. The origin is the bottom vertex farthest to the right; therefore, the baseline case of no disclosure is represented by the line of slope 1 in the back face on the right. To see these plots for prevalence reduction, refer to *SI Appendix*, Fig. S2. An animation showing the dependence of year-end prevalence on p and s he initial twenty years of disclosure is available at https://bedbugdisclosure.shinyapps.io/shinyapp/.

infestation ($1/\gamma$), and vacancy multiplier (b). We recalculated the cost of disclosure while varying each parameter across the range given in Table 1 and holding all other parameters constant at their estimated values. Estimated cost and final prevalence were insensitive to k (*SI Appendix*, Figs. S6 and S7), more sensitive to $1/\gamma$ (*SI Appendix*, Fig. S8), and most sensitive to b (*SI Appendix*, Fig. S9). For example, doubling the baseline value of k led to little change, even if we assume that b is much higher (5 vs. 1.3) so that relocation transmission is responsible for ~50% of total transmission. Doubling the length of infestation $1/\gamma$ yielded similar prevalence at year 20 and savings that were 60% larger. However, doubling the amount by which infestation increases move-out rate (b) reduced prevalence at year 20 by a factor of 2 and increased savings by a factor of 2.7; this result suggests that the predictions at the baseline value of b are conservative.

These sensitivity results should not be interpreted as contradicting a key analytical result above: that policies which would change the treatment rate would have a large impact on bed bug prevalence. Our model was implemented by calibrating the β parameter to a value that would give the desired baseline prevalence after all other parameter values (including k, γ, and b) had been assigned. Thus, our sensitivity analysis explored how our estimates of disclosure cost are affected by inaccuracies in these input parameters given a fixed prevalence.

The values of the cost parameters used to calculate vacancy, treatment, and turnover costs (Table 1) can vary by geographic region and can influence the impact of disclosure policies on landlords. Overall, in regions with lower monthly rents, but no difference in treatment or turnover costs, the initial costs of disclosure for landlords will be lower and will convert to savings more quickly. The converse is true for regions with higher rents. Even rents at the 95th percentile of those reported in the American Community Survey (~$2,000) lead to savings within ~7 y (40). Results for alternative costs can be explored with our online tool: https://bedbugdisclosure.shinyapps.io/shinyapp/.

Impact of Intermarket Migration. To determine how a rental market with legislated disclosure policies might be impacted by surrounding markets that do not adopt such policies, we considered a model that relaxes the assumption of a closed population and includes immigration of new tenants from external markets with a stable bed bug endemic. This model, outlined in *SI Appendix*, assumes that a fraction, i, of new tenants moving into vacant units come from external markets that have a net bed bug prevalence e. First, we assumed that the prevalence of bed bug infestation in the external market (e) was 5% and examined the effect of disclosure on cost and bed bug prevalence ranging the external tenant fraction (i, the proportion of new rentals that are taken by external vs. internal tenants) from 0 to 40%. Second, we assumed that 20% of new rentals were by tenants from an external market ($i = 0.2$) and then evaluated cost and prevalence for a range of possible levels of infestation prevalence in these immigrant tenants (e from 5 to 20%). As expected, we found that migration into the system decreased the eventual savings caused by disclosure policies but that these savings were still apparent after approximately 5 y and significant after 10 y in all cases (*SI Appendix*, Fig. S10 *A* and *C*). Similarly, migration from regions with stable infestation levels dampened the decline in prevalence under disclosure policies, particularly when bed bug prevalence in the external market was much greater than the baseline prevalence of the system. For instance, prevalence declined to only 3.7% after 10 y when 20% of new tenants were immigrants from an external market with 20% prevalence, compared with 2.7% in the reference case with no immigration (*SI Appendix*, Fig. S10*D*). When the external prevalence is comparable to the baseline prevalence of the system (e = 5%), intermarket migration had less impact on prevalence decline, even with a large fraction of immigrant tenants (i = 40%; *SI Appendix*, Fig. S10*B*).

Impact of Disclosure in a Structured Population. Our model so far has assumed that the population of rental units susceptible to

infestation is uniform and well-mixed. However, it is also possible that certain subsets of the population play an outsized role in sustaining the epidemic, perhaps due to inability to afford prompt treatment or more frequent movement between apartments. Infestation prevalence in such hypothetical subpopulations might be more difficult to control with disclosure or other policies, and the cost and benefit estimates could be different. Constructing a realistically heterogeneous model of bed bug transmission and control is difficult, given the very limited surveillance data, so we created simple two-population models to evaluate some worst-case scenarios using methods detailed in *SI Appendix*. In each model, a small, high-prevalence subpopulation is completely disconnected from a larger, lower-prevalence population, and the high-prevalence subpopulation can sustain higher infestation rates by having a lower treatment rate γ, a higher move-out rate m, or a higher "aversion to bed bugs" (higher b and k). We found, in each scenario, that landlord costs remained positive and did not convert to savings in the high-prevalence subpopulation. These results show that if infestation is extremely concentrated in one segment of the population, landlords serving that population will bear most of the short-term costs and reap most of the long-term benefits of disclosure policies—but that sometimes savings never occur. This major difference occurs because effective R_0 values may be much higher in these high-prevalence subpopulations, meaning that control measures need to be much more severe to significantly reduce infestation levels. Disclosure policies would likely need to be combined with other interventions, such as decreasing time to treatment in high-prevalence subpopulations.

Discussion

The spread of bed bugs is a growing concern in cities around the world, and despite the obvious similarity to infectious diseases and the need for evidence-based control policies, bed bugs have received little attention from mathematical models (41). In this work, we introduce a simple model that incorporates the major defining features of bed bug spread. We track the dynamics of both the housing market and infestation prevalence, since the interaction between these two processes, which occur on similar timescales, is a key feature of bed bug outbreaks. Tenant turnover contributes to the spread of infestation between rental units, and infestations can lead to increased rates of vacating apartments and reluctance to move into units with a history of infestation. Our model explicitly considers two general modes by which bed bugs can spread between units—either by tagging along with tenants who leave infested units for new ones or by importation into bug-free occupied units—and provides a framework to estimate the relative contribution of these processes based on individual parameters describing components of human or bed bug behavior. This model can be used as a framework for evaluating proposed control measures.

In response to the recent resurgence of bed bug infestations, some states and municipalities have adopted bed bug disclosure laws that require landlords to disclose the infestation histories of their units to all potential tenants [New York City (16)] or to tenants upon request [San Francisco (17), Mason City (18), Maine (20), and Connecticut (19)]. These policies seek to protect tenants, but some fear that such measures would impose costs that would unfairly punish landlords. We used our model of bed bug transmission to evaluate the potential impact of disclosure policies on bed bug prevalence and cost to property owners. Overall, we found that the financial impact of disclosure to landlords varies over time: beginning as a net cost, but peaking quickly and subsequently falling continuously. Contrary to fears that disclosure policies would create significant economic burden to landlords, we predict that, in many scenarios, they are likely to result in significant savings over relatively short time horizons.

Our results suggest that the magnitude of the initial cost, and the eventual savings, are driven by two key factors: the prevalence of bed bug infestations before disclosure and renter selectivity. When prevalence is relatively high (closer to ~10% vs. ~1–2%), implementation of disclosure creates more vacant units initially but eventually leads to significant savings as prevalence drops. Renter selectivity is a theoretical value that would depend, in part, on knowledge and attitudes toward bed bugs and also, in part, on the supply and demand for rental units in a regional market. In cities with an abundance of rental units, renter selectivity might be higher because in these "renters' markets," renters can afford to be more choosy. On the other hand, in cities like New York City and San Francisco, where there is a relative housing shortage and demand for rental units is high (42, 43), renter selectivity is expected to be low. It is perhaps not a coincidence, then, that it is in these cities that bed bug disclosure has been legislated in some form, since the immediate economic risk to landlords is likely to be low.

Although our work focused primarily on the financial impact of disclosure on landlords, we also found disclosure to benefit tenants. Infestations can be a costly ordeal for tenants, even when landlords bear sole financial responsibility for extermination. Costs to tenants can include replacing furniture and so-called do-it-yourself treatments, which may result in property or bodily damage (12, 35, 44). Frustration with the inability to eliminate bed bug infestations can lead tenants to vacate their homes, sometimes breaking their lease to do so (36). The decrease in prevalence that is likely to result from well-enacted disclosure policies would intuitively benefit tenants, whom we expect to experience savings from the first year of policy implementation. Moreover, these benefits are likely to extend beyond the rental market to private homeowners, who may acquire infestations from renters, and local governments, some of which are very large landlords of public housing (45).

We found, using reasonable parameter regimes and assuming a baseline steady-state prevalence of <10%, that the basic reproductive ratio, R_0, of bed bug infestations is close to 1. Consequently, small perturbations to the system, due to disclosure or other policies, can push bed bug populations toward local elimination (since $R_0 > 1$ is needed for persistence). Despite significant uncertainty surrounding several model parameters, there is good reason to believe that this finding is accurate. Bed bug populations were easily eliminated on a grand scale following the availability of dichlorodiphenyltrichloroethane (DDT) and other synthetic insecticides in a way that other household pests, such as cockroaches, were not (46). If R_0 of bed bug infestations were much greater than one, it is highly unlikely that their populations would have crashed so dramatically. It is equally unlikely that it would have taken so long for bed bugs to reemerge following their development of resistance to DDT and pyrethroid insecticides [which was reported as early as 1948 (47)].

An important assumption we make in calculating β, and hence R_0, is that the prevalence of bed bug infestations reported from field studies represents a system at or near equilibrium. While this assumption is supported by general patterns present in internet search patterns (*SI Appendix*, Fig. S11), these have their own complex dynamics that may not directly mirror the prevalence of infestations (48). If bed bug infestations are indeed continuing to climb, and we have underestimated the infectivity, the break-even timepoint, at which the cost of disclosure equals the savings due to decreased prevalence, would be delayed. If the true equilibrium prevalence of infestation exceeds a threshold (~16% based on our best estimates of the parameters), the break-even point could be delayed indefinitely (*SI Appendix*, Fig. S12). However, savings can be recovered if disclosure is more effective at averting tenants from infested units than we estimated ($s \to 1$) or if disclosure policies also improve treatment rates.

Due to the relative dearth of data on bed bug infestations, our model was formulated under a few simplifying assumptions, and our results should be interpreted in the context of these limitations. Our model does not incorporate elasticities that are likely to exist in the rental-housing market. Landlords might prefer to lower the rent of disclosed units rather than let them sit vacant, and not allowing prices to respond to disclosure may lead us to overestimate vacancy costs and, potentially, the decrease in prevalence due to the quarantine effect. Our model is most relevant to a single segment of a rental market in which units, landlords, and tenants are expected to be relatively similar. Results from our metapopulation models, which considered two hypothetical subpopulations that exist in isolation, suggest that disparities in the populations of interest that are not accounted for in our mass-action model could lead us to overestimate the benefits of disclosure; however, some benefits are likely to be recovered with more realistic levels of intermediate mixing between groups. We did not consider more complex metapopulation or network structures beyond our two-population models, and it is unclear before parametrizing such models how their results might diverge from those obtained from our mass-action model (49).

While our model included a single value for the treatment rate (γ), it was able to at least partially account for variability in treatment time that could result from some infestations being intrinsically more difficult to detect or treat; this property follows from the formulation of our model as a system of ordinary differential equations, which makes the implicit assumption that treatment times are exponentially distributed (and thus have a long tail). However, our model does not include temporal dynamics and feedback effects on γ. Disclosure policies require the disclosure of treatment along with infestation histories and would likely put pressure on landlords to treat vacant units before showing them to potential tenants. Not capturing these changes in γ as landlords respond to disclosure may lead us to underestimate the benefits of disclosure. On the other hand, our model does not and cannot anticipate the evolution of additional insecticide resistance in bed bug populations, which could have enormous effects on γ and the future of the epidemic as a whole. Additional model limitations, along with possible extensions, are presented in *SI Appendix*.

Despite recent advances in pest-management strategies (34, 50) and improvements to urban housing, bed bugs have reemerged as a household pest and public health concern. Our model provides a first step toward evidence-based prospective analysis of policies to control the spread of bed bugs. Our results show that bed bug control is a classic collective action problem: Individual landlords bear the initial costs of disclosure policies, but after a few years, both landlords and tenants will benefit from the reduction in prevalence of infestations. Additionally, we show that while bed bugs are extremely difficult to eliminate from homes, they are likely to be less difficult to control in cities. We predict that, on average, a single infested residence infests little more than one additional residence ($R_0 \gtrsim 1$), whether by infectious or relocation transmission. Consequently, rational and enforced policies have great potential to stem the bed bug epidemic.

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Isolation, identification, and time course of human DNA typing from bed bugs, *Cimex lectularius*

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ABSTRACT

Bed bugs (*Cimex lectularius* L.) are ectoparasitic wingless insects that feed on the blood of mammals, typically in residential settings. The objectives of this study were to establish a time-course of human DNA quantitation from bed bugs and to generate human DNA profile(s) of a host and/or multiple hosts from a bed bug that fed on human blood. Female human genomic DNA concentrations ranged from 18.370 to 0.195 ng/bed bug at 0–108 h post blood meal (PBM), male human genomic DNA concentrations ranged from 5.4 to 0.105 ng/bed bug at 0–108 h PBM, and pooled human female and male blood ranged from 5.49 to 0.135 ng/bed bug at 0–96 h PBM. Human autosomal STR complete profiles were obtained until 72 h PBM for female, male, and pooled human blood. These results reveal that identification of multiple human hosts is possible from a single bed bug. However, the ratio of each contributor may be variable depending on the amount of blood ingested from each individual and the time difference of blood consumed from each subject. Average peak heights for three STR markers of low (D3S1358), medium (D13S317), and high molecular weight (D2S1338), were also compared over time. Peak heights were consistently higher for the low molecular weight marker over all time intervals. These data suggest that some markers can be successfully recovered more than three days PBM. Hence, bed bugs can serve as physical evidence in temporal and spatial predictions to match suspects and/or victims to specific locations in criminal investigations.

1. Introduction

Bed bugs (*Cimex lectularius* L.) are ectoparasitic insects that feed on the blood of mammals and are typically found in groups within residential settings [1]. The enormous global increase in bed bug infestations in the last two decades can be attributed to increased international commerce and travel, popularity of second-hand furniture and thrift shops, and insecticide resistance. Bed bugs usually aggregate in close proximity to sleeping and resting areas such as beds, sofas, and recliners. They are easily translocated by passive dispersal and adapt to multiple host species [1–4].

The recent resurgence of bed bugs, especially in North America, has garnered interest in the field of forensics. Identifying an individual based on blood or tissue isolated from insects can be used to implicate a suspect in the time and place of a crime. However, human DNA isolated from an insect would have to be (1) stable and intact long enough to be useful in a forensic investigation and (2) unambiguously identifiable to an individual host. Researchers have successfully isolated, amplified, and profiled human mitochondrial DNA (mtDNA) from blood-feeding insects, including bed bugs [5] and human crab louse (*Pthirus pubis*) [6], and from maggots of the shiny blue bottle fly (*Cynomyopsis* [=*Cynomya*] *cadaverina*) feeding on human tissues [7,8]. Additional research has been conducted to isolate and profile human nuclear DNA from the blood meals of other insects such as human lice (*Pediculus humanus capitis*) [9] and mosquitoes (Culicidae) [10], and from maggots [11–14].

Experiments with the human crab louse (*P. pubis*) and human head louse (*P. humanus capitis*), both obligate human ectoparasites, found that blood-fed lice and their excreta could yield DNA from single or multiple human hosts [6,9,15]. Human DNA has been extracted and identified from individual mosquitoes for up to 15 h post blood meal (PBM) [16] and subsequent studies have confirmed the sensitivity and reproducibility of using DNA

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fingerprinting of mosquito blood meals to identify individual human hosts [17]. Short Tandem Repeat (STR) identification was used to determine the feeding preferences of *Aedes aegypti*, the yellow-fever mosquito and vector of dengue virus, thus demonstrating the role played by a migrating population in spreading the virus [18,19]. A more recent study determined that Culicinae mosquitoes can be relevant to a criminal investigation when present at a crime scene, as human DNA could be successfully typed from these insects 56 h after a blood meal had been taken [20]. In another case, almost all 15 STR loci were generated in a victim's profile obtained from a mosquito discovered at the crime scene, confirming the presence of the victim in close proximity to the suspect [10]. Lastly, human STR profiles could be obtained from maggots feeding on corpses 2–4 days later depending on the level of starvation the maggots had experienced [11]. Furthermore, it is suggested that STR profiles were best obtained when the maggots were immediately preserved after feeding [14].

The forensic use of bed bugs has several advantages, compared with other blood-feeding arthropods. Bed bugs are wingless insects; therefore, they remain in close proximity to their feeding location. Although a small fraction of a bed bug population can walk relatively long distances, most remain near the host. Hence, any long-range displacement, especially between buildings, can be attributed to human-mediated translocation. This makes bed bugs useful for validating the location of a suspect or a victim in legal and/or forensic investigations. Bed bugs are hemimetabolous, like lice, so all life stages feed on blood, whereas only adult female mosquitoes and fleas (holometabolous) do so. This increases the probability of finding some recently fed bed bugs. Bed bugs also feed infrequently, generally every 4–7 days, which allows genotyping single host blood DNA profiles. In addition, bed bugs have a relatively long lifespan and may survive for longer periods without blood meals compared to mosquitoes, fleas, and lice.

Szalanski et al. [5] demonstrated that DNA could be isolated from recently blood-fed bed bugs and it is qualitatively sufficient for DNA genotyping. However, to date, there are no documented reports about successful human blood identification and/or full human STR typing from a bed bug fed on human female, male, or pooled (female:male) blood. Moreover, it is not known whether the identification of multiple humans can be made from a bed bug that had fed on multiple hosts such as male/female, male/male, and female/female combinations.

The objectives of this study were to establish a time-course for human blood identification, to quantify human DNA from bed bugs, and to generate DNA profile(s) of a host and/or multiple hosts from a bed bug that fed on human blood. We demonstrate that sufficient quality and quantity of human blood and human DNA can be isolated and typed from a bed bug. Hence, bed bugs can serve as physical evidence both in terms of temporal and spatial predictions for criminologists to match suspects and/or victims to specific locations in criminal investigations and homeland security.

2. Materials & methods

2.1. Human blood feeding to bed bugs

C. lectularius colonies of the Harold Harlan strain (HH; also known as Fort Dix, collected in 1973), were maintained in an incubator at 27 °C, ~50% relative humidity, and 12:12 h light:dark in the Urban Entomology lab at North Carolina State University. Colonies were fed defibrinated rabbit blood in an artificial feeding system maintained at 37 °C, as described in Romero and Schal [21]. Newly emerged adult bed bugs were starved for two weeks to clear the rabbit blood and guarantee complete engorgement on human blood. Starved adult males were separated into three feeding containers and allowed to feed in the dark on human defibrinated blood (Bioreclamation IVT, New York, NY). Bed bugs in the three different containers were fed female human blood (n = 100), male human blood (n = 100), and pooled blood (1 female:1 male, vol:vol) (n = 90). Bed bugs were given 30 min to feed, and fully fed adult males (Fig. 1) were individually placed into labeled 2.0 mL Eppendorf tubes. Ethanol (95%) was added to each Eppendorf tube at 12 h intervals PBM, beginning at 0 h (immediately after feeding) up to 108 h, and tubes were stored at −80 °C. The samples were then transported to Fayetteville State University (FSU), and stored at −80 °C in the Forensic Science laboratory for further testing.

2.2. Isolation of blood from bed bugs

Ethanol was removed from the tube, leaving only the engorged bed bug. Approximately 500 μL of dH_2O was added to the tube. To remove traces of ethanol, the tube was gently vortexed. Distilled water was removed from the tube, and the cleaning process was repeated twice more. After the bed bug was cleaned, 10 μL of Rapid Stain Identification (RSID) kit universal buffer was added to the tube. A clean probe was used to vigorously homogenize the bed bug. Once homogenized, all fluid containing blood (approximately 10 μL) was removed from the tube and placed onto a labeled Flinders Technology Associates (FTA) blood card (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Special care was taken to ensure that cuticle fragments were excluded from the fluid. This process was repeated twice more for each bed bug sample. When the last of the fluid from the bed bug sample tube was blotted, the FTA blood card was placed inside a sterile hood in the dark to air dry overnight.

2.3. Isolation of DNA from FTA cards

Each FTA card containing the dried blood from a single bed bug was cut into smaller pieces and transferred into a labeled 2 mL Eppendorf tube. Each FTA card sample, including reference female and male blood samples, was soaked in 500 μL of digestion buffer (10 mM Tris-HCl, 10 mM EDTA, 50 mM NaCl, 2% SDS and 20 μg/mL proteinase K) and incubated at 56 °C for a minimum of 3 h. As a quality control measure, a reagent blank, negative control, and positive control were included. Subsequently, DNA was extracted from each sample using phenol: chloroform: isoamyl alcohol (25:24:1) [22]. The aqueous phase of the DNA extraction was transferred to an Amicon® Ultra-4 Centrifugal Filter Device (Millipore, Billerica, MA) for DNA concentration, and the manufacturer's recommended protocol was followed. The tube contents were washed twice with TE buffer (10 mM Tris HCl, 10 mM EDTA, pH 8) and centrifuged at 5000 g for 15 min. The recovered DNA, in approximately 15–20 μL, was stored at −20 °C.

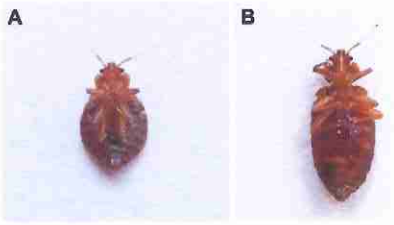

Fig. 1. Photographs depicting bed bugs: (A) nonengorged, unfed bed bug (B) engored, fully fed bed bug on human blood.

2.4. DNA quantitation

Total human genomic DNA as well as human male DNA were quantified from each bed bug using the Quantifiler® Duo Kit (Thermo Fisher Scientific, Waltham, MA) run on the ABI 7500 Real-Time PCR System (Thermo Fisher Scientific). The manufacturer's validated quantitation protocol was followed.

2.5. DNA amplification and genotyping

Polymerase Chain Reaction (PCR) reactions were prepared according to the manufacturer's recommended protocol using the AmpFlSTR® IdentiFiler® PCR Amplification Kit (Thermo Fisher Scientific). The DNA template was 1 ng in a final volume of 25 μL for each PCR reaction. PCR tubes were loaded into a 96-Well GeneAmp® PCR System 9700 (Bio-Rad, Hercules, CA) for amplification. All amplification reactions were accompanied by negative and positive controls.

2.6. Human specific STR genotyping

Following PCR amplification, the ABI PRISM® 310 Genetic Analyzer (Thermo Fisher Scientific) was employed for electrophoretic separation of amplified products. For ABI 310 sample preparation, 24.5 μL Hi-Di™ Formamide (Thermo Fisher Scientific), 0.5 μL GeneScan™ 500 LIZ® Size Standard (Thermo Fisher Scientific), and 1 μL of PCR amplified product or AmpFlSTR® Identifiler™ Allelic Ladder (Thermo Fisher Scientific) were added to each sample. The reaction tubes were heated at 95 °C for a 3 min denaturation step, immediately snap-cooled on a freezer block for 3 min, and then subjected to capillary electrophoresis. The samples were separated on a 47 cm × 50 μm capillary tube (Thermo Fisher Scientific).

Amplified products were electrokinetically injected for 5 s and fractionated on an ABI Prism® 310 Genetic Analyzer using POP-4® Polymer (Thermo Fisher Scientific). Data were analyzed using a peak detection threshold of 100 relative fluorescence units (RFU) for all dyes with GeneMapper® ID v3.2.1 software (Thermo Fisher Scientific).

Human autosomal STR alleles generated from each bed bug at 0–108 h PBM were divided by the total number of alleles from the reference (female and/or male) blood samples. For example, at 0 h female blood fed bed bug gives 16 STR alleles plus an amelogenin, gender marker, totaling 17 alleles. This number (17 alleles) is divided by the total number of alleles from the respective female reference sample alleles, which are 29 human autosomal STR alleles plus X, an amelogenin marker, totaling 30 alleles. Hence, the percentage of correct alleles at 0 h female PBM is (17/30) × 100 = 56.6%.

The Institutional Review Board (IRB) proposal for this study was approved by the FSU Human Rights in Research Committee (IRB # 2013-P-0039).

3. Results

3.1. Recovered liquid from fed bed bugs

The volume of liquid recovered from each bed bug approximately represented the volume of human blood and bed bug blood. Freshly fed and fully engorged bed bugs fed on female, male, or pooled human blood yielded 3–4 μL. The post-feeding volume dropped to 1–2 μL after 60 h.

3.2. Human genomic DNA quantitation from bed bugs

Human genomic DNA concentrations for female DNA ranged from 1.225 to 0.013 ng/μL, with a total amount of 18.370– 0.195 ng of human genomic DNA per bed bug (ng/bed bug) at 0–108 h PBM. Male human genomic DNA concentrations ranged from 0.36 to 0.007 ng/μL, with a total of 5.4–0.105 ng/bed bug at 0–108 h PBM and pooled human (female and male) blood ranged from 0.366 to 0.009 ng/μL, totaling 5.49–0.135 ng/bed bug at 0–96 h PBM (Table 1). Average total human DNA recovered from a bed bug fed on female, male, and pooled blood trend shows that it is highest at 12 h from bed bugs fed on female and male blood but highest at 24 h from bed bugs fed on pooled blood. Subsequently average total DNA concentration starts declining with a few inconsistencies. Interestingly, the average total human DNA recovered from a bed bug fed on human female blood is 3.4–5.5 times higher than human DNA recovered from bed bugs fed on human male and pooled blood (Table 1). Pure human defibrinated female and male blood was used as reference.

3.3. Human specific STR genotyping

Human specific STR genotyping profiles were generated from amplified human genomic DNA isolated from human blood obtained from each bed bug. Human autosomal STR profiles from each bed bug sample were compared with reference female and male blood DNA autosomal STR profiles for complete and/or partial match or no profile at various 12 h intervals PBM. Percentage of correct autosomal STR alleles were calculated by counting all the human autosomal STR alleles successfully amplified from the bed bug (0–108 h at 12 h, PBM intervals) and divided by the total number of alleles from the respective reference sample. Complete human female blood fed bed bugs were the only samples that generated autosomal STR profiles that matched with female

Table 1
Human DNA recovered from a bed bug fed on human female, male, and pooled (female and male) blood at 12 h intervals from 0 h–108 h with reference (female and male) samples. Pure human defibrinated female and male blood was used as reference.

Time post blood feeding sacrifice of bed bug (Hrs)	Isolated DNA volume (μL)	Average human female DNA (ng/μL)	Total human female DNA from a bed bug (ng/Bb)	Average human male DNA (ng/μL)	Total human male DNA from a bed bug (ng/Bb)	Average pooled human DNA (ng/μL)	Total pooled human DNA from a bed bug (ng/Bb)
0	15	0.202	3.035	0.111	1.665	0.019	0.278
12	15	1.225	18.370	0.36	5.4	0.220	3.306
24	15	0.965	14.480	0.155	2.325	0.366	5.49
36	15	0.741	11.110	0.21	3.15	0.196	2.94
48	15	0.502	7.526	0.149	2.235	0.075	1.131
60	15	1.118	16.765	0.075	1.119	0.072	1.082
72	15	0.267	4.012	0.014	0.206	0.043	0.645
84	15	0.048	0.718	0.041	0.615	0.011	0.167
96	15	0.152	2.284	0.007	0.106	0.009	0.137
108	15	0.013	0.198	0.012	0.186	N/A	N/A
Reference blood	15	1.4	21	0.71	10.65	1	15

reference autosomal STR profiles until 96 h PBM (Fig. 2). However, 72 h PBM male and pooled samples generated completely concordant profiles with the respective reference samples (Fig. 2). Though complete profiles were not consistently obtained, partial profiles were successfully obtained from female and male fed blood samples for all time intervals and for pooled samples until 96 h PBM (Fig. 3).

Average peak heights for three STR autosomal markers of low (D3S1358), medium (D13S317), and high molecular weight (D2S1338), were also compared over time (Fig. 4). Peak heights were consistently higher for the low molecular weight marker compared to the medium and high molecular weight markers (D13S317 and D2S1338) over all time intervals. These data suggest that some markers can be successfully recovered more than three days PBM; however, most success will be found using smaller autosomal STR markers.

4. Discussion

This study confirms the application of forensic DNA methods for the recovery of good quality and sufficient quantity of human blood from bed bugs fed on either female, male, or pooled blood. The ability to generate human STR profile(s) depends on the volume of the initial blood ingested and the rate of its breakdown in the bed bug. We assert that bed bugs may be better suited for human genotyping than other blood-feeding species found at crime scenes due to their larger post-feeding volume, feeding and digestion kinetics, life cycle and ecology. Bed bugs take infrequent large blood meals punctuated with relatively long periods of starvation [1]. Therefore, starving bed bugs for two weeks to maximize the volume of their blood meal was consistent with their normal ecology. The volume of a fully engorged bed bug on the artificial feeding system was 3.92 ± 0.21 μL (SEM) [23] whereas sand flies (*Phlebotomus argentipes*) ingest only 0.63–0.73 μL (20), and the volumes of blood meals in engorged mosquitoes range between 2–3 μL [24,25]. Cat flea (*Ctenocephalides felis*) females, which are common indoor pests, ingest about 1 μL each during a 3 h feeding trial [26]. Moreover, unlike other blood-feeding arthropods in the indoor environment, all life stages of the bed bug feed on host blood, and while adult females ingest larger amounts of blood, juveniles would ingest appreciably smaller volumes of host blood. Also, unlike other blood feeders, fully engorged bed bugs can use a single blood meal to mature to their next life stage and to produce eggs for 10–15 days [27]. Therefore, the likelihood of extensive blood mixing from different hosts is lower than with other blood feeders.

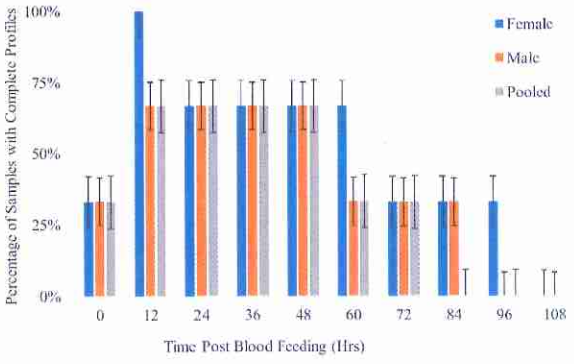

Fig. 2. Percentage of complete human autosomal STR profile matched with female, male, and pooled blood DNA extracted from bed bugs. Data below (100 RFU) analytical threshold is included. Bars represent standard error of the mean (SEM).

Importantly, changes in the volume of ingested blood cannot be accurately inferred from a simple homogenization of the bed bug because this procedure does not differentiate the bed bug's own blood and water content from host blood. The critical consideration for forensic investigations, however, is not the volume of the PBM host blood but its DNA content, quality, and detectable human specific STR markers. Our results show that human DNA content and quality decline dramatically over time PBM, as with other blood feeding insects. Thus, our ability to generate human DNA profile from bed bugs depends not only on the size of the ingested blood, but the time-course of its digestion in the bed bug. Crime scene technicians should be trained to recognize the difference between an engorged and nonengorged bed bug (Fig. 1), because recently fed bed bugs provide better quality and quantity of human DNA.

We have found that the ability to obtain readable human DNA fingerprints from bed bugs depended on the kinetics of its digestion. To understand the blood meal digestion rate in bed bugs, and hence, to understand how long DNA profiles can be obtained that match a reference and/or search in a database, we analyzed three representative autosomal STR markers; D3S1358, D13S317, and D2S1338 are low, medium, and high molecular weight IdentiFiler® STR markers, respectively. Peak heights at these markers demonstrated the degradation of DNA over time (Fig. 4). As expected, the high molecular weight marker, D2S1338 showed higher rates of degradation over time compared to the medium (D13S317) and low molecular weight markers (D3S1358) (Fig. 4). The degradation rate was similar in female, male, and pooled blood PBM.

The goal of this study was to establish positive human identification from a bed bug at 12 h intervals PBM until 108 h. Our data suggest that correct (100%) positive identification can be established up to 96 h PBM from bed bugs fed on female blood, up to 84 h PBM from bed bugs fed on male blood, and up to 72 h PBM from bed bugs fed on pooled blood (Fig. 2). Presumably, the combined activities of digestive enzymes and nucleases degraded the ingested human pooled blood so that complete human autosomal STR profiles could not be obtained after 72 h from pooled PBM and 84 h from human male PBM. However, ingested female blood did not show a complete drop out of the STR markers until 108 h PBM, 24 h longer compared to male and 36 h longer compared to pooled PBM, respectively. This difference should be viewed with caution until replicated with multiple cohorts of bed bugs and multiple sources of human blood, including naturally fed bed bugs. Nevertheless, these results might suggest that the rate of human blood digestion might differ depending upon the source of the host blood. Future investigations are warranted to determine whether various blood types impact bed bug feeding preferences, ingested blood volume, and rate of digestion.

The low percentage (~55%) of correct allele calls immediately after the ingestion of blood (0 h) for female, male, and pooled human blood corresponded to low amounts of human DNA recovered (Table 1) from the bed bug (average female blood = 0.202 ng/μL; average male blood = 0.111 ng/μL; and average pooled blood = 0.019 ng/μL). This low amount of DNA obtained at 0 h is in contrast to results obtained from maggots, where optimal DNA genotyping results occurred for maggot samples that were immediately preserved after feeding [14]. The low DNA recovery in this study could be attributed to partial inhibition of DNA polymerase during and shortly after feeding. This unknown inhibitory factor would later (after 12 h) become inactivated, yielding higher amounts of DNA (Table 1) and higher percentage of correct alleles (Fig. 3). This time-course suggests that perhaps salivary secretions associated with the blood-feeding event might be involved. PCR inhibitors have been detected in the heads of various insect species, and it is often recommended that heads be

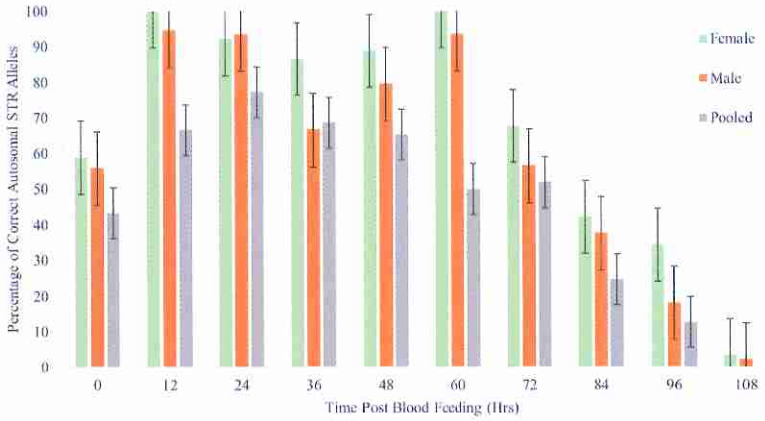

Fig. 3. Percentage of correct autosomal STR allele calls for female, male, and pooled blood extracted from bed bugs. Data below (100 RFU), analytical threshold is included. Bars represent standard error of the mean (SEM).

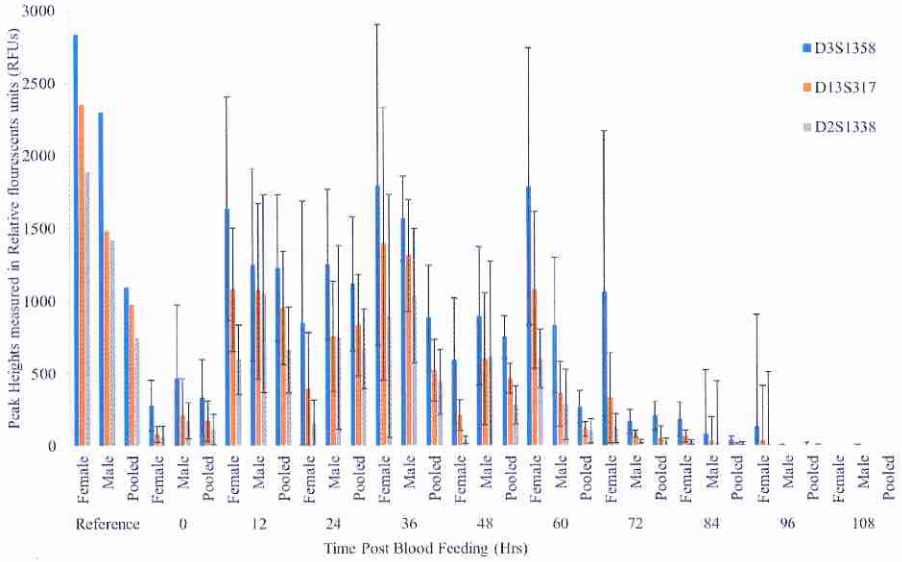

Fig. 4. Average peak heights at low (D3S1358), medium (D13S317), and high (D2S1338) molecular weight autosomal STR markers for female, male, and pooled bloodmeal samples. Data demonstrates the degradation of DNA over time. Data below (100 RFU) analytical threshold is included. Bars represent standard error of the mean (SEM).

removed for PCR reactions that do not require the head [28]. We are investigating if this low amount of human male-specific, Y chromosome DNA as well as low percentage of correct Y STR allele calls are observed at 0 h PBM in bed bugs fed on human male blood.

A second goal of this study was to determine if human blood meal sources could be identified from a mixture of host blood. Bed bugs, unlike some other blood-feeders, are quite stealthy feeders. They feed at night while the host sleeps, and usually complete a blood meal in a single feeding bout with no disturbance to the host. At times, however, feeding may be disrupted, and the bed bug may resume feeding on a different host, resulting in pooled blood from two hosts within the bed bug. Previous research demonstrated that human DNA was successfully detected up to 20 h after blood consumption by two body lice fed on pooled blood meals [9]. Here we have demonstrated that a mixed DNA profile can be obtained after feeding pooled blood from a female host and a male host to a bed bug. The mixed profiles matched with their reference blood profiles up to 72 h post feeding (Fig. 2). These results indicate that identification of multiple human hosts is possible from a single bed bug. However, the ratio of each contributor may be variable depending on the amount of blood ingested from each individual and the time difference of blood consumed from each subject. With all the advantages bed bugs have over other hematophagous insects used for human identification, crime scene technicians should strive to locate and collect them for use as physical evidence in forensic investigations.

5. Conclusion

This study demonstrates for the first time that the use of forensic biology and entomology methods enable identification of human blood sources from bed bugs. It is also evident that single source as well as multiple human hosts can be positively genotyped up to 72 h PBM. However, DNA degradation is a concern as time elapses; therefore, we recommend that crime scene technicians collect multiple blood fed bed bugs from the crime

scene to maximize the recovery of human blood and human-specific DNA and increase the chances of generating complete DNA profile(s) from bed bugs.

Authors contributions

This is a brief detail of each author's contribution in the research design, methods development, methods validation, data production, data analysis, literature review, manuscript writing, edits, revision, and project administration. curation

1. **Coby Schal, Ph.D.:** Dr. Lodhi had the original research idea using bed bugs for human identification. He presented the experimental design and details of research and requested Dr. Schal's collaboration for feeding human blood to bed bugs at Dr. Schal's entomology laboratory at North Carolina State University. Dr. Schal agreed and provided bed bugs for preliminary experiments. Hence, Dr. Schal was involved from the original conceptualization and experimental design. He contributed in data analysis, data interpretation, manuscript writing, revision, and project administration.
2. **Natalia Czado, MS:** Ms. Czado was involved in data analysis, figures preparations, data interpretation, manuscript writing, and revision.
3. **Richard Gamble, BS:** Richard was involved during the DNA quantitation, DNA amplification, figures preparations, and data analysis.
4. **Amy Barrett, BS:** Amy was involved during the experimental designs and validation. Amy later helped in data collection, figures preparations, data analysis, and literature review.
5. **Kiera Weathers, BS:** Kiera was involved during the experimental designs and validation. She helped in data collection, figures preparations, data analysis, references citation, and literature review.
6. **Khalid Mahmud Lodhi, DSc.:** Dr. Lodhi had the original research idea using bed bugs for human identification. He presented the experimental design and details of research and requested Dr. Schal's collaboration for feeding human blood to bed bugs at Dr. Schal's entomology laboratory at North Carolina State University. Therefore Dr. Lodhi's role included funding acquisition, conceptualization, experiment design, methods validation, experimental work, data analysis, data interpretation, manuscript writing, edits, revision, and project administration.

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RESEARCH ARTICLE

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Comparison of different cytogenetic methods and tissue suitability for the study of chromosomes in *Cimex lectularius* (Heteroptera, Cimicidae)

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Abstract

In the article we summarize the most common recent cytogenetic methods used in analysis of karyotypes in Heteroptera. We seek to show the pros and cons of the spreading method compared with the traditional squashing method. We discuss the suitability of gonad, midgut and embryo tissue in *Cimex lectularius* Linnaeus, 1758 chromosome research and production of figures of whole mitosis and meiosis, using the spreading method.

The hotplate spreading technique has many advantages in comparison with the squashing technique. Chromosomal slides prepared from the testes tissue gave the best results, tissues of eggs and midgut epithelium are not suitable. Metaphase II is the only division phase in which sex chromosomes can be clearly distinguished. Chromosome number determination is easy during metaphase I and metaphase II. Spreading of gonad tissue is a suitable method for the cytogenetic analysis of holokinetic chromosomes of *C. lectularius*.

Keywords

holokinetic chromosomes, spreading method, squashing method, testes, midgut, karyogram

cally important species. This genus is characterised by possession of the all-important heteropteran cytogenetic features: holokinetic chromosomes (e.g. Wolf et al. 1997, Mola and Papeschi 2006, Papeschi and Bressa 2006, Guerra et al. 2010, Poggio et al. 2014), achiasmatic male meiosis of collochore type (Nokkala and Nokkala 1983, Nokkala and Grozeva 2000, Grozeva and Nokkala 2002, Ituarte and Papeschi 2004, Grozeva et al. 2008, 2010, Poggio et al. 2009, 2014, Kuznetsova et al. 2011), postreductional inverted male sex chromosome meiosis (Viera et al. 2009, Kuznetsova et al. 2011), and the diffuse stage (Kuznetsova and Maryańska-Nadahowska 2000, Bressa et al. 2002b, Rebagliati et al. 2005, Lanzone and Souza 2006). However the cytogenetic research on *Cimex* species is difficult because of some other chromosome characteristics, such as the small size, high morphological similarity and superspiralization during almost the whole period of chromosomal division (e.g. Ueshima 1966, Manna 1984). Holokinetic chromosomes lack a primary constriction and thus a localized centromere, which facilitates structural rearrangements of the karyotype by nonlethal chromosomal fusions and fragmentations. Fusions in this type of chromosomes do not result in dicentric chromosomes. Chromosome fragments are able to attach to spindle fibres and migrate normally during mitosis or meiosis, which enables them to go through further cell division (e.g. Motzko and Ruthmann 1984, Howe et al. 2001, Mandrioli and Manicardi 2003, Schvarzstein et al. 2010).

In addition to the above mentioned features, the important human ectoparasite model species *Cimex lectularius* Linnaeus, 1758 shows intraspecific variability in number of sex chromosomes from three (X_1X_2Y) to 21 (X_1X_2Y+18 extra Xs) (e.g. Darlington 1939, Slack 1939, Ueshima 1966, Sadílek et al. 2013). In the family Cimicidae, the sex is determined by the presence of an XX/XY (female/male) simple sex chromosome system in 53 cytogenetically analysed species. Most cimicid species, including the majority of *Cimex* species, also possess a multiple sex chromosome system $X_1X_1X_2X_2$/X_1X_2Y (except *C. antennatus* Usinger & Ueshima, 1965, *C. latipennis* Usinger & Ueshima, 1965 and *C. incrassatus* Usinger & Ueshima, 1965 with the basic XX/XY system) (Poggio et al. 2009, Grozeva et al. 2010, Kuznetsova et al. 2011, Sadílek et al. 2013). Four cimicid species possess constantly three X chromosomes ($X_1X_2X_3Y$, male) (*Paracimex capitatus* Usinger, 1966, *P. inflatus* Ueshima, 1968, *P. philippinensis* Usinger, 1959 and *Hesperocimex coloradensis* List, 1925) and two species four X chromosomes ($X_1X_2X_3X_4Y$, male) (*Cimex adjunctus* Barber, 1939 and *C. brevis* Usinger & Ueshima, 1965) (Ueshima 1979, Kuznetsova et al. 2011).

Intraspecific variability in the number of X chromosomes has been described in three cimicid species from the subfamily Cimicinae, *Paracimex borneensis* Usinger, 1959 (2X; 5-9X), *P. capitatus* (2-6X) and *C. lectularius* (2-20X) (summary in Ueshima 1966, 1968, 1979). The numbers of *C. lectularius* X chromosomes can differ among different populations (localities), or among specimens within one population. Even a single specimen can contain cells with different numbers of X chromosomes (Ueshima 1966, 1979, Sadílek et al. 2013). Preliminary study has also indirectly indicated the possibility of a variable number of X chromosomes in an obligatory bat parasite *Cimex pipistrelli* Jenys, 1839 (Sadílek et al. 2013). Therefore, it seems possible that intraspe-

Results and discussion

Hotplate spreading

The basic principle of the hotplate spreading technique is to turn extracted tissue into a suspension and let cells to adhere to the surface of a microscope slide (optimal is SuperFrost quality slide) as the drop was moved on the slide by pushing it with fine tungsten needles and evaporated. The resulting semipermanent slide (without cover slip) is characterized by its long durability (for years), stored at 4 °C for basic Giemsa staining or -20 °C to -80 °C for further molecular analysis (e.g. FISH). *Cimex lectularius* specimens were dissected in hypotonic solution 0.075 M KCl immediately after killing, to keep the gonad tissue hydrated and remove debris of other tissues. During hypotonisation, the cells receive additional water due to osmosis, making them larger, the contents of the cell are loosened and chromosomes become more individualized. Chromosomes can be damaged or washed away during final dissociation in case of excessive hypotonic treatment. However, chromosomes are still too compact and are not analysable in insufficiently hypotonised cells. Several time periods of tissue hypotonisation were tried: 10, 15, 20, 25 and 30 minutes. The best results were obtained from samples after 25 minutes of fresh hypotonic solution treatment.

Tissue fixation in methanol: glacial acetic acid 3:1 was the next step, methanol can be replaced by 99.9% ethanol. Alcohol causes immediate death of cells and acetic acid penetrates the membrane for quick ideal preservation of inner structures especially chromosomes. Two types of fixation were tested, one step fixation for 5, 10, 15 or 20 minutes, and two step fixation for 5+10, or 10+20 minutes. However, the duration of fixation had a minor effect on the final quality of chromosomes on slides. Two step fixation for 5+10 minutes was found to be optimal, the tissue dehydration effectiveness increased because in the second fixation step dilution by water from hypotonic solution was reduced to minimum.

Fixed tissue was mechanically suspended on the slide with tungsten needles and cells were chemically released by adding of 1–2 drops of 60% acetic acid. Undissociated clusters of tissue were removed. The slides with suspension were put on a warm (45 °C) histological plate and the drop was moved all around the slide with the needle. Adhering cells can create hardly diagnosable clusters without that movement. The chromosome sets are very often overlapping in those clusters. Suspension movement also contributes to evenly distributed chromosomal material on the slide surface. The slides were stained on the second day, allowing them to dry properly and to avoid loss of chromosomes. The staining was carried out using a 5% Giemsa solution in Sörensen phosphate buffer (pH = 6.8) for 10, 15, 20, 30 or 40 minutes (optimum in 30 minutes). The stained slides were stored in a refrigerator at 4 °C. The mechanism of cell adherence is described in detail by Imai et al. (1988).

The squashing technique is the more widely used method in Heteroptera cytogenetics. Usually, living specimens are directly fixed in ethanol: glacial acetic acid or methanol: glacial acetic acid (3:1) and can be stored at 4 °C for later use. Dissected gonad tissue is squashed under a cover slip in a drop of 45% acetic acid, which is then frozen using dry ice (solid CO_2) (e.g. Kuznetsova and Nadachowska 2000, Grozeva

Tissue suitability and results obtained

Cimex lectularius reproduction is acyclic, which is why it is almost impossible to find out the exact age or physiological condition of wild specimens. Negative results from specimens with inactive gonads (absence of cell division) could be caused just by starving. Exact age and condition could be known only in laboratory reared specimens and it is also possible to use eggs or larvae of specific age.

Chromosome slides were made from tissues with the highest mitotic index, which express amount of dividing cells. Meiotic chromosomes could be isolated only from gonad tissue, but mitotic chromosomes should be obtained from all types of proliferating tissues as in insect e.g. hemolymph, epithelium of digestive tract and in holometabolous insect imaginal disc.

Gonads. Generally, tissue of gonads is used for cytogenetical studies, mainly testes (Fig. 1A, D) (e.g. Kuznetsova et al. 2004, Bressa et al. 2009, Grozeva et al. 2010, Poggio et al. 2011, 2014), sometimes ovaries (Fig. 1B, C, E) (e.g. Angus et al. 2004, Waller and Angus 2005). We obtained chromosomes in all various stages of spermatogenesis (mitosis and meiosis) from *C. lectularius* testes, and only mitotic chromosomes in its ovaries. However, also frequent meiotic pachytene cells (Fig. 2E) were recorded in ovaries. This could mean that the female pachytene is a prolonged resting phase when immature oocytes stop meiosis until feeding or mating. In the contrast to females, the pachytene stage in *C. lectularius* males is very short and its finding is extremely rare.

Gonads from 4th and 5th instar larvae were analyzed as well as those of adults (Fig. 1A–C). Gonads of the 4th larval instar are always very small, any manipulation of them is quite difficult as well as a correct determination of sex. Size of the 5th larval instar gonads (Fig. 1D, E) can be different in wide spectrum, from miniature as in the 4th larval instar to large and well developed in sub adult specimens, in which also sex can be distinguished easily. In the older 5th instar larvae, nuclei from mitosis to meiotic metaphase II can be seen (Fig. 2A, B, L).

In *C. lectularius* feeding directly initiates mating behaviour and cell division in gonads, thus this is a required condition for gonad growth and gamete production (Usinger 1966). In our study, small gonads and therefore negative slides were recorded even from recently (approximately 7 days) fully engorged specimens, which probably could not digest their meal and start gamete production.

Testes tissues were shown to be very suitable for the *C. lectularius* cytogenetic research. They contain large quantities of cells in all stages of meiotic and mitotic division and provide enough information for complete karyotype analysis. Ovarian tissue is suitable in cases of lack of males or as a reference in samples with a higher chromosomal variability, and to confirm the sex chromosome system in comparison with chromosomes of males. In samples of *C. lectularius* with variable karyotype, it is interesting to observe complementarity of chromosome number between males and females, and it is also possible to study females with varying X chromosome numbers in oocytes, originating from breeding of specimens with different karyotypes (Sadílek et al. 2013).

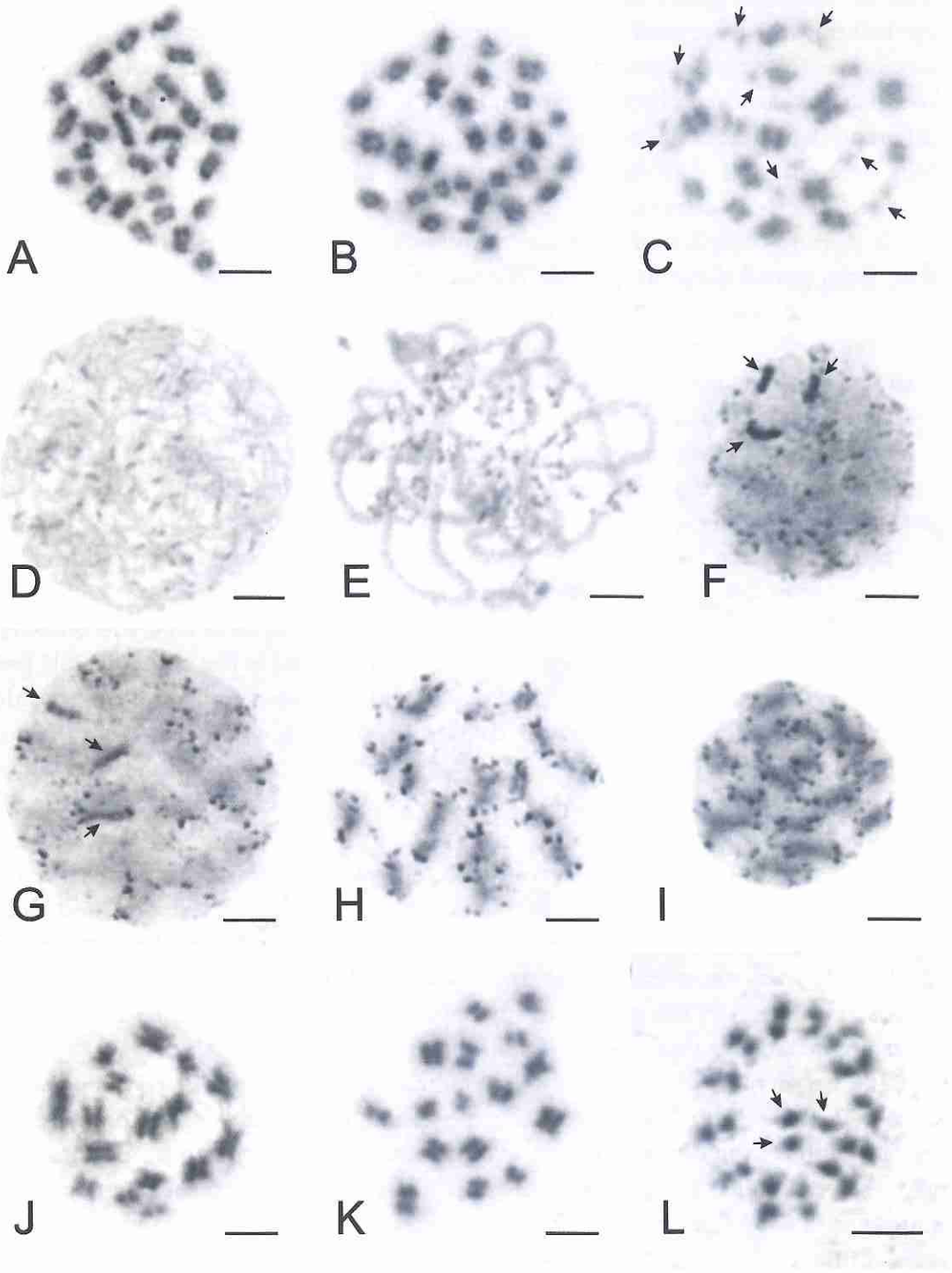

Figure 2. Various stages of mitotic and meiotic *Cimex lectularius* chromosomes with basic karyotype 2n = 26+X_1X_2Y (**A, B, D–L**) and karyotype 2n = 26+$X_{1-10}Y$ (**C**), stained with Giemsa. **A** Mitotic prometaphase ♂ **B** Mitotic metaphase ♂ **C** Metaphase I ♂ **D** Leptotene ♀ **E** Pachytene ♀ **F** Diffuse stage ♂ **G** Diffuse stage - postpachytene transition ♂ **H** Postpachytene ♂ **I** Late postpachytene ♂ **J** Prometaphase I ♂ **K** Metaphase I ♂ **L** Metaphase II ♂. Arrow = sex chromosome (**F, G, L**) or fragments of supposedly sex chromosomes (**C**). Scale bar = 5 μm.

Chromosome division phases studied

The following stages of cell division were observed with various frequencies in *C. lectularius* males. Mitotic cells were recorded especially in metaphase and prometaphase stages (Fig. 2A, B) in 80% of specimens. Leptotene and pachytene stages were detected only in two specimens. In late prophase I, the most frequent meiotic cells were diffuse stage in 90% of specimens and postpachytene in 30% of specimens (Fig. 2F–I). Less frequently, cells in metaphase I (Fig. 2K) were observed in 25% of specimens, and cells in metaphase II (Fig. 2L) were the most rare, only in 20% of specimens. Metaphases I were frequently very abundant in the specimens, in a contrast short lasting stages of prometaphase I (Fig. 2J) and II were observed always in small amounts and only in a four specimens.

On slides from ovary cells in mitotic metaphase stage (100% of specimens) only early prophase I (leptotene and pachytene) (Figs 2D, E) from meiotic division were detected. Leptotene nuclei were recorded only in 10% of specimens, pachytene nuclei were observed in 50% of specimens in small densities only. In females no cells were observed in late meiosis, which was the main stage of male cells.

Leptotene (Fig. 2D) and pachytene (Fig. 2E) nuclei are isopycnotic and did not show any distinct features. At diffuse stage (Fig. 2F), autosomes are decondensed and the sex chromosomes are distinctly positive heteropycnotic. During transition from diffuse stage to postpachytene (Fig. 2G), the sex chromosomes become isopycnotic and cannot be distinguished from autosomes. Postpachytene may be considered as meiotic prophase stage that substitutes diplotene and diakinesis in organisms with achiasmatic meiosis where no recombination occurs. During postpachytene, autosomes condensate again and dark terminal spots on telomeric regions of each chromatid appear (Fig, 2G–I). The dark spots disappear at the end of postpachytene, and from prometaphase onwards the chromosomes are isopycnotic (Fig. 2J) and continue in condensation until metaphase I.

In metaphase I (Fig. 2K), nucleus with basic karyotype 2n = 26+X_1X_2Y shows 13 autosomal bivalents and three sex chromosomes, which do not pair with each other. Male metaphase II is radial, the 13 autosomes dispose in a ring configuration and the X_1, X_2 and Y chromatids form a pseudotrivalent, which lies at the centre of it (Fig. 2L), in concordance with observation of Ueshima (1967), Grozeva et al. (2010) and Sadílek et al. (2013). Metaphase II is the only stage in which it is possible to definitely distinguish autosomes and sex chromosomes. The chromosome arrangement of metaphase II precisely matches the inverted meiosis of sex chromosomes, in which reductional division of autosomal bivalents occurs in anaphase I whereas the sex chromosomes segregate chromatids (equational division). In anaphase II, autosomes segregate sister chromatids, and the X_1 and X_2 chromatids segregate from the Y (Ueshima 1966, 1979, Grozeva et al. 2010), even with 20 X supernumerary sex chromosomes (Sadílek et al. 2013) (Fig. 2C). Only the metaphase II reflects clearly number of sex chromosomes in *C. lectularius* with supernumerary sex chromosome fragments, because this is the only phase where autosomes and sex chromosomes can be distinguished.

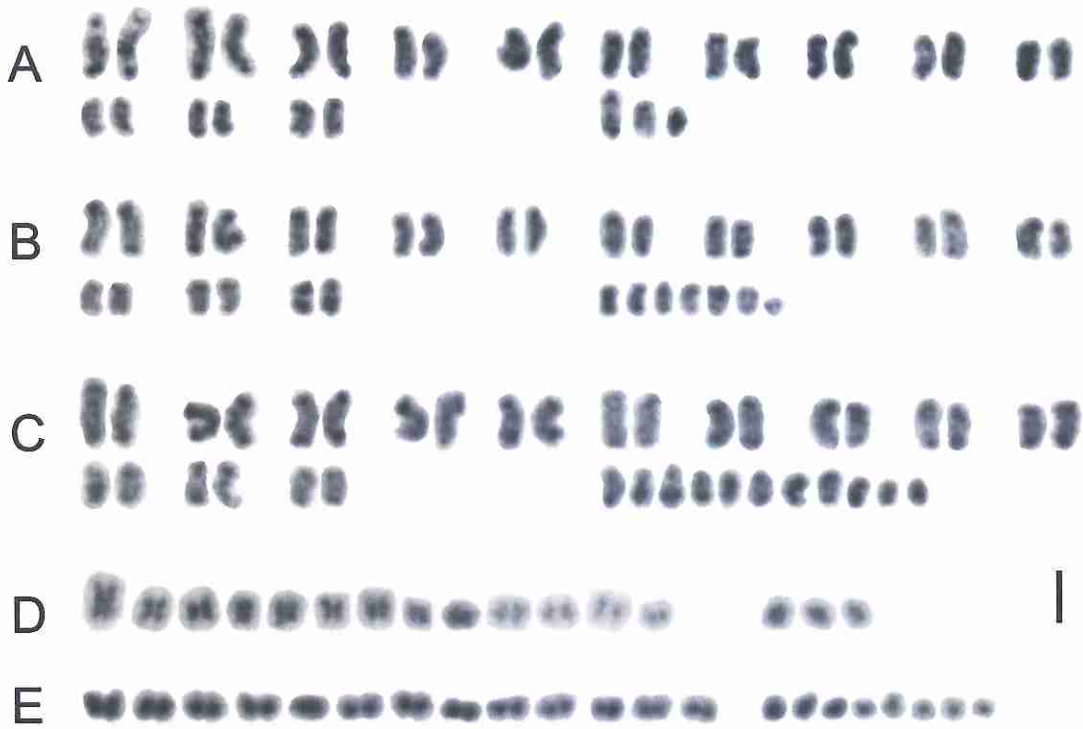

Figure 3. Male mitotic and meiotic karyograms of *Cimex lectularius* chromosome variants. **A-C** Mitotic prometaphase. **A** 2n = 26+X_1X_2Y **B** 2n = 26+$X_{1\text{-}6}Y$ **C** 2n = 26+$X_{1\text{-}10}Y$ **D** Prometaphase II, 2n = 26+X_1X_2Y **E** Metaphase II, 2n = 26+$X_{1\text{-}7}Y$. Scale bar = 5 μm.

In each of *C. lectularius* karyotypes the size of chromosomes gradually decreases. That is a reason why the size expressed only as a percentage is not very suitable for karyotype comparison among congeneric species with different diploid chromosome numbers because they have different distribution of length. However, in case of *C. lectularius* chromosome fragments we can predict their very small size as on example of metaphase I with 2n = 26+$X_{1\text{-}10}Y$ (Fig. 2C). In the contrast two karyograms show additional sex chromosomes (Fig. 3B, C) of almost the same size as the sex chromosomes in karyotype with basic chromosome number (Fig. 3A). It suggests an occurrence of non-disjunction or even possibility of chromosome fragments different spiralization. Another explanation could be the fragments resulted from fragmentation in different parts of the original sex chromosomes. If fragments origin is independent in various populations, they simply cannot be identical and must differ by size and content. All these hypotheses need further study. The karyogram assembly brought us at least rough chromosome size comparison of *C. lectularius* various karyotypes.

Conclusion

The hotplate spreading technique has many advantages in comparison with the squashing technique. It is suitable for use by cytogenetic beginners as they need only to

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Distribution and host relations of species of the genus *Cimex* on bats in Europe

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Abstract. The species of the genus *Cimex* (Heteroptera: Cimicidae) are important ectoparasites of European bats. Unlike other ectoparasites, they are attached to the body of their host only when they need to feed, otherwise they stay in refugia in bat roosts. Consequently, they are often overlooked by bat specialists and in many countries they are either unknown or poorly characterized. This study reports results from thorough investigations of bat roosts of diverse bat species in a Northwest-Southeast transect across Europe: Czech and Slovak Republics, Hungary, Serbia and Bulgaria. The distribution of *Cimex lectularius* follows the synanthropic habitats of its principal hosts, *Myotis myotis* and *M. emarginatus*, both Mediterranean elements of the European fauna. The climate in natural roosts (i.e. caves) inhabited by these bats in southern areas appears to restrain the presence of cimicids. In central Europe, *C. pipistrelli* parasitizes, beside *M. myotis*, many crevice-dwelling bat species indigenous to the boreal zone. However, in southern Europe, it appears only in connection with *Nyctalus noctula*. *C. lectularius* was confirmed for five host bat species and newly recorded for *Rhinolophus ferrumequinum*, *C. pipistrelli* was confirmed for seven bat species and newly recorded for *Myotis nattereri*. The first record of *C. emarginatus* outside of its type locality and *Myotis alcathoe* as a new host are reported. The host preferences of the species of the genus *Cimex* are discussed.

Key words: biogeography, faunistics, ectoparasites, ecology, host-parasite relationship

Introduction

Because their social behavior, bats constitute a particularly favourable environment for diverse fauna of ectoparasites (Marshall 1982). Bat aggregations have allowed bugs of the family Cimicidae (Heteroptera) to develop a unique exploitation strategy. No life stage of cimicids is permanently attached to the body of a bat. Both larvae and adults remain hidden in refugia in bat roosts and use the hosts only in order to feed on their blood (Usinger 1966). However, it is also likely that cimicids actively search for host as means of dispersal (Heise 1988, Balvín et al. 2012b).

The impact of cimicids on bats can manifest itself as an increase in self-grooming (Bartonička 2008). Such discomfort makes the colony of "crevice-dwelling" bats (i.e. bat species with a habit to roostin tight crevice spaces) switch roosts (Bartonička & Gaisler 2007, Bartonička & Růžičková 2013). This reduces the abundance of cimicids, but can also promote their spread to other suitable bat roosts. Colonies of philopatric species of bats, which have a habit to roost in large open spaces, i.e. attics in central Europe (reffered as "attic-dwelling" bats elsewhere in this paper) respond by moving within these spaces which are often large enough to escape the reach of cimicid refugia (Bartonička & Růžičková 2012). Furthermore, cimicids are vectors of diverse bat pathogens or can cause secondary infections (Williams et al. 1976, Bowers & Woo 1981, Adelman et al. 2013).

The diversity of Cimicidae comprises 110 known species classified in 24 genera and six subfamilies (Henry 2009). About two thirds of the species are

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associated with bats, which were suggested to be the original host of the family (Horváth 1913). The remaning species are related to birds. Three bat-associated species, including the bed bug *Cimex lectularius* Linnaeus, 1758, have adopted humans as another host.

The European fauna of cimicids is represented by the genera *Cimex* and *Oeciacus*, which were deemed synonymous (Balvín et al. 2013, 2015), and the recently discovered *Cacodmus vicinus* Horváth, 1934 (Quetglas et al. 2012). Species of the former genus *Oeciacus* are parasites in nests of birds of the family Hirundinidae. Since bats may occupy these nests (Loye 1985, Schulz 1995, Ritzi et al. 2001), *Oeciacus* bugs are occasionally found on bats as well (Rotschild 1912, Ritzi et al. 2001). However, there are three strictly bat-associated *Cimex* species in Europe: *Cimex lectularius*, *C. pipistrelli* Jenyns, 1839 and *C. emarginatus* Simov, 2006.

The lineages of the bed bug *C. lectularius* specialized to people and bats are completely isolated and, as a result, morphologically and genetically distinct (Balvín et al. 2012a). The population parasitizing on bats has historically been considered a separate species (Poppius 1912). Recent evidence is consistent with this (Booth et al. 2015). Thanks to human migration, the bed bug became cosmopolitan. However, little is known about the original distribution of the bat-associated population. The bed bug has been reported on several bat species (Table 1) but can be regarded as common only in roosts of *Myotis myotis*. Bed bugs are also found quite often in roosts of *M. emarginatus*. In central Europe, the two species usually roost in spacious attics. In southern Europe, these bats roost in caves that are too humid and cold for cimicids (Simov et al. 2006). The synanthropy of bats probably developed only in the last several centuries (Horáček 1983). Before then, bed bugs might have inhabited caves in the Middle East, as suggested by Povolný & Usinger (1966), who published the only report of a bed bug in the natural habitat of a cave (Afghanistan). The only other records from bats in this region are by Abul-Hab (1979), from mist-netted *Pipistrellus kuhlii* in Iraq and possibly a meadow in Iran (Golestan province, 37°22′1″ N 55°59′3″ E, 27 May 2006, A. Reiter and P. Benda lgt.; assigned as bat-associated bed bug based on morphology by Balvín et al. 2012a). It is possible that the European population of the bed bug found on bats in the present study is of rather recent origin. Furthermore, this bed bug population appears to be discontinuous from the original population inhabiting caves.

The validity of two of the former three European species of the *C. pipistrelli* group was recently dismissed (Balvín et al. 2013). Though the taxonomy has not been completely resolved, only one species is likely to exist in Europe and only one is therefore recognized in this study. Morphological variation in diagnostic characters was connected with association to different bat species. Since the taxonomy of the other seven species of the *C. pipistrelli* group described from Asia is based on similar metric characters, the situation found in European taxa also makes the validity of this taxonomy questionable. Therefore, any conclusions regarding the distribution of taxa from the *C. pipistrelli* group are not currently possible. It is clear, however, that the species group is the dominant cimicid parasite of bats in the Palaearctic region. While the records of *C. lectularius* on the crevice-dwelling bat species like *Nyctalus* and *Pipistrellus* spp. are sporadic, these bats are common hosts for *C. pipistrelli* (Povolný 1957, see Table 1 for other references). *C. pipistrelli* is common in roosts of *Myotis myotis* as well (e.g. Lederer 1950, Usinger 1966). In contrast to *C. lectularius*, because of its association with *Nyctalus* spp., the area of *C. pipistrelli* in Europe stretches as far as the southern peninsulas (Lanza 1999, Simov et al. 2006, Balvín et al. 2012b).

Cimex emarginatus was known only from the type locality, a roost of *M. emarginatus* in a building near Primorsko, Bulgaria (Simov et al. 2006). Recently, this species was confirmed to be distinct from but related to *C. lectularius* based on mtDNA data (unpublished), as Simov et al. (2006) presumed.

This paper reviews the records of bat-associated *Cimex* species from bat roosts in Europe made during collection of material for population genetic studies (Balvín et al. 2012a, Balvín et al. 2013, Booth et al. 2015). The geographic distribution of the two species in Europe is reviewed. Also, their host and habitat preferences are discussed.

Material and Methods

In the Czech Republic, a systematic monitoring of three species of bats (*R. hipposideros*, *M. myotis* and *M. emarginatus*) included in the Natura 2000 system is carried out (Bartonička & Gaisler 2010). Roosts of other bat species are also occasionally visited or searched for. The localities monitored in the Czech Republic are maternity colonies consisting of females bearing their young in the summer shelters. They are mostly located in buildings. The material reported in this study has been mostly collected from such summer roosts by the authors by joining the monitoring teams,

Table 1. Review of records of *Cimex lectularius* and *C. pipistrelli* for European bat species in the literature and our material. Records from *Pipistrellus kuhlii* are from Iraq. The first reference for each bat species known to us is listed. For details on our records from roosts see Supplementary material Table 2. Country codes: BG – Bulgaria, CZ – Czech Republic, FI – Finland, FR – France, GE – Germany, HU – Hungary, CH – Switzerland, RS – Serbia, SK – Slovakia, UK – United Kingdom. The single record for *Rhinolophus ferrumequinum* refer to a case when hosting cimicids was directly proved; however, *Rhinolophus* spp. are often found in colonies mixed with usual hosts of cimicids where they can serve as substitution hosts as well. Bold records refer to newly recorded host-parasite relationships. * - roost shared with *M. myotis*. The names *M. blythii* and *M. oxygnathus* may be synonymous.

Bat species	*Cimex lectularius*		*Cimex pipistrelli*	
	Reference	No. of roosts in our material	Reference	No. of roosts in our material
Eptesicus serotinus	Baagøe 2011	2 (CZ, HU)	Southwood & Leston 1959	
Myotis bechsteinii	Scheffler 2008		Morkel 1999	
Myotis blythii	Tagilcev 1971 (*Cimex* sp.)		Tagilcev 1971 (*Cimex* sp.)	1*(HU)
Myotis brandtii			Heise 1988	1 (CZ)
Myotis dasycneme			van Rooij et al. 1982	
Myotis daubentonii	Wagner 1967		Heise 1988	2 (CZ), 1 (GE)
Myotis emarginatus	Usinger & Beaucournu 1967	3 (CZ), 2 (HU), 4 (RS), 2 (SK)	Usinger 1966	2*(HU, SK)
Myotis nattereri				**1 (CZ), 1 (GE)**
Myotis myotis	Povolný 1957	23 (CZ), 3 (GE), 5 (SK), 3 (FR, CH, HU)	Lederer 1950	41 (CZ), 2 (HU), 9 (SK), 2 (FR, CH),
Myotis mystacinus	Poppius 1912		Kerzhner 1989	
Myotis oxygnathus	Usinger 1966	1*(HU)		
Nyctalus lasiopterus			Balvín et al. 2012b	
Nyctalus leisleri	Bobkova 2001		Nelson & Smiddy 1997	
Nyctalus noctula	Heise 1988		Povolný 1957	3 (CZ), 4 (BG, GE, SK, UK)
Pipistrellus kuhlii	Abul-Hab 1979		Abul-Hab & Shihab 1990	
Pipistrellus nathusii			Heise 1988	
Pipistrellus pipistrellus	Rybin et al. 1989		Jenyns 1839	
Pipistrellus pygmaeus			Bartonička 2007	3 (CZ), 1 (UK)
Pipistrellus sp.		2 (CZ), 1*(SK)		3 (UK)
Plecotus auritus	Balvín et al. 2012b			
Rhinolophus ferrumequinum		**1*(RS)**		
Vespertilio murinus	Dubinij 1947		Horváth 1935	

starting 2005. Some material of cimicids has also been collected by the monitoring specialists during the colony censuses.

The data from Bulgaria were collected 1) during a thorough survey targeted at cimicids, covering about 500 roosts between 1997 and 2008 and 2) during the course of a project entitled "Mapping and identification of conservation status of natural habitats and species" (Phase I in Natura 2000 zones in Bulgaria), covering about 1600 diverse bat roosts since 2011.

In Serbia, a systematic monitoring of mixed maternity roosts of *Myotis emarginatus* and *Rhinolophus ferrumequinum* has been carried out since 2003 in the area of southern Banat, Vojvodina province. Six roosts have been found and shown to communicate with each other based on capture-recapture experiments. In addition to these, about 500 roosts of bats, mainly of species *Myotis capaccinii*, *M. myotis*, *M. oxygnathus*, *Miniopterus schreibersii*, *Nyctalus noctula* and *Pipistrellus pipistrellus*, often mixed with each other

or *Rhinolophus* spp., are known over the entire area of Serbia and examined for the presence of ectoparasites, though not as regularly as those in Vojvodina province or Bulgaria.

Finally, about 10 days of field work in bat roosts in Slovakia and Hungary were carried out. Part of the material was collected by bat specialists in other European countries (see Supplementary material Table 2). If possible, the close surroundings of the bat colonies were examined for the presence of cimicids, paying special attention to the most likely shelters of their refugia: crevices in walls and wood below and around the bat colony, or bottom side of objects located below the colony. In some spacious roosts, the colony was unreachable and only the guano and surrounding floor could be inspected. If unsuccessful, dead cimicids were searched for in the guano or spider webs. The number of female bats was noted. Maximal number of cimicids were collected, or, at least dead individuals and exuviae; however the collection had often to respect the need to keep the presence of bugs from the wardens of the respective buildings.

Results and Discussion

Host relations

The records of *Cimex lectularius* and *C. pipistrelli* in bat roosts in Central Europe, Serbia, Bulgaria and some other European countries are summarized in Table 1 and Supplementary material Table 2. Generally, the number of colonies monitored annually varied among bat species. It was high in non-dwelling bats with high fidelity to shelters, such as *Rhinolophus hipposideros*, *Myotis myotis* and *M. emarginatus*. In species with low philopatry the long-term monitoring is difficult. Even in common species (e.g. *Pipistrellus pipistrellus*, *Nyctalus noctula*) it is difficult to locate summer colonies. If found, the roosts cannot be checked in the following years as they are often destroyed or the bats relocate during large-scale renovations of buildings (especially prefabricated houses). Furthermore, such roosts are often difficult to check for both bats and cimicids because of their crevice character, in contrast to the spacious roosts of philopatric bat species. Therefore, the numbers of records for each bat species are not representative with regard to the incidence of cimicids in their roosts. However, the incidence can be considered high at least in some species like *Nyctalus* spp. and *Pipistrellus* spp., given the number of records on mist-netted individuals (Balvín et al. 2012b) or roosts inhabited by these species (Supplementary material Table 2).

Cimex pipistrelli was confirmed as a parasite of the attic-dwelling bat species *M. emarginatus*, *Myotis myotis* and, possibly, *M. blythii*, as well as the crevice-dwelling bat species *Myotis brandtii*, *M. daubentonii*, *Nyctalus noctula*, *N. leisleri*, *Vespertilio murinus*, *Pipistrellus pygmaeus* and, possibly, *P. pipistrellus*. It was newly recorded for *Myotis nattereri* (Lužnice, Czech Republic, Supplementary material Table 2). The new record for *Nyctalus lasiopterus* was already mentioned by Balvín et al. (2012b).

Cimex lectularius was confirmed to parasitize on the attic-dwelling bat species *Myotis myotis*, *M. emarginatus* and, possibly, *M. oxygnathus*, as well as the crevice-dwelling bat species *Eptesicus serotinus* and *Pipistrellus* sp. (see below).

Among attic-dwelling bat species, both cimicid species were shown to be very common in roosts of *Myotis myotis* in central Europe. Less than 25 % of these synanthropic roosts inspected by the authors were negative. In the Czech Republic, out of about 140 monitored roosts about 80 were inspected for the presence of cimicids, which were found in 64 (~80 %) roosts. In total, *Cimex lectularius* was recorded in 36 roosts of *M. myotis* and *C. pipistrelli* in 46. No cimicids were found in the cave roosts in Serbia and Bulgaria.

Myotis myotis was the only bat species hosting both cimicid species in one roost, though only in few isolated cases. Mixed infestation was found only at the colony in Dubá (Czech Republic) in 2013 and 2014, whereas in 2009 only *C. lectularius* was discovered (Supplementary material Table 2). In 2009, this bat colony was complemented by several hundred bats from a nearby roost in Doksy. In this roost, only *C. lectularius* was found in the year the bats moved to Dubá, so other, unknown bats had to have brought *C. pipistrelli*. A mixed infestation was also revealed found in Zemianske Kostoľany (Slovakia), but among remains of bodies of unknown age. A change of infestation from *C. pipistrelli* to *C. lectularius* over years has likely been recorded in Úštěk and Držovice (Czech Republic). However, samples from these roosts were not numerous and may not reflect the composition of the species. Part of samples from other roosts also contained only a few individuals but comparing the numbers of roosts with consistent and inconsistent record of *Cimex* species it is likely that populations of cimicids in single roosts mostly consisted only of one species. Based on this limited evidence, the mechanisms of coexistence or competition between *C. pipistrelli* and *C. lectularius* can only be speculated on. However, the co-occurrence of the two species is likely caused by the co-occurrence of different bat species in a single roost. Attic-dwelling bats like *M. myotis*, frequent

hosts of *C. lectularius*, often share attics with crevice-dwelling bats like *Pipistrellus* spp. (e.g. Host'ovce) or *Eptesicus* spp. (e.g. Oleksovice; see Supplementary material Table 2 or Czech bat Conservation Society database, unpublished), frequent hosts of *C. pipistrelli*. *Myotis oxygnathus* was confirmed as a host of *C. lectularius* only in a colony mixed with *M. myotis* (Martonyi, Hungary, Supplementary material Table 2). In the same area of the Aggtelek Karst in Hungary, two more roosts inhabited only by *M. oxygnathus* were visited and no trace of cimicids was found. As *M. oxygnathus* is physically and ecologically almost identical to *M. myotis*, the absence of cimicids in the roosts of this species may not be a coincidence, considering the at least 80 % incidence of cimicids in synanthropic *M. myotis* roosts in our record. It is possible that *M. oxygnathus* does not constitute a suitable host for cimicids for an unknown reason.

In roosts of *M. emarginatus*, only *C. lectularius* was confirmed. It was found in 11 synanthropic roosts, often shared with *Rhinolophus* spp. A similar number of roosts with similar microclimates was negative. There is a single published record of *C. pipistrelli* from *M. emarginatus* (Usinger 1966), while the records of *C. lectularius* are at least three (Usinger & Beaucournu 1967, Protić & Paunović 2006). *C. pipistrelli* was found only in two roosts of *M. emarginatus* shared with *M. myotis*. It is possible that *M. emarginatus* is the only host that *C. lectularius* does not share with *C. pipistrelli*, at least in Central Europe and Serbia.

Further south, however, *M. emarginatus* has been shown to host *C. emarginatus* (Simov et al. 2006). The colony from which the type material originated flew away in 2005. Since then, only a single record (female) of *C. emarginatus* is available from mist-netted *Myotis* cf. *alcathoe* (Bulgaria, Monastery Sveti Archangel, Malashevska planina Mts., Blagoevgrad district, 41°51′23.04″ N, 22°59′31.92″ E, 10 September 2011, B. Petrov, I. Alexandrova lgt.). If the determination of the bat species is correct, it is also the first record of a cimicid for this newly described species. In 2006, *C. emarginatus* was unsuccessfully attempted to be confirmed by investigating a large unfinished building of a hotel near the type locality in Primorsko, which was inhabited by multiple colonies of *M. emarginatus* and many other bat species (*Myotis blythii*, *Myotis* sp., *Miniopterus schreibersii*, *Rhinolophus* spp., see Benda et al. 2003). Only two specimens of *C. pipistrelli* were collected in this study (Supplementary material Table 2).

The presence of *C. lectularius* in roosts of *Pipistrellus* sp. was shown indirectly. The bugs attacked people in a gamekeeper's house near Hnanice, South Moravia, and a hunting hide nearby. In both buildings, colonies of *Pipistrellus* sp. were recorded (*P. pipistrellus* or *P. pygmaeus*; not distinguished at that time). Although these bugs were unusually small, similar to *C. pipistrelli* from *Pipistrellus* spp., in other morphological characters they clearly corresponded to *C. lectularius* from other bat species (Balvín et al. 2012a). The third record of *C. lectularius* from *Pipistrellus* sp. was made from a roost shared with *M. myotis* (Host'ovce, Slovakia).

Roosts inhabited only by *Rhinolophus* spp. were always free of cimicids, which is consistent with the literature. Unlike vespertilionid bats, *Rhinolophus* spp., at least in the synanthropic roosts in Central Europe, rarely form tight clusters (Gaisler 1966) and readily move through the roosting space. This likely makes them an unsuitable host for cimicids. Until recently, *Plecotus* spp. was similarly considered an unsuitable host for cimicids, regarding the entire lack of published records for such common bats. Unlike *Nyctalus* or *Pipistrellus* spp., most of the maternity colonies of *Plecotus* spp. are comprised of only a few individuals (Anděra & Horáček 2005), which were believed to be incapable of hosting a population of cimicids, similar to the diffusive colonies of *Rhinolophus* spp.

Out of the 140 summer roosts that are annually monitored in the Czech Republic, at least 23 are inhabited by species that commonly host cimicids together with *Plecotus* or *Rhinolophus* spp. (Bartonička & Gaisler 2010). *Rhinolophus* spp. are also listed as hosts of cimicids in records from colonies mixed with, for example, *Myotis emarginatus* (Usinger 1966, Usinger & Beaucournu 1967, Protić & Paunović 2006). A recent record of *C. lectularius* has been made from *Plecotus auritus* (Balvín et al. 2012b). Furthermore, the first record of *Cimex* (*C. lectularius*) from bats from Serbia was made from *Rhinolophus ferrumequinum* caught by harp trap. More recently, *C. lectularius* was found on three more individuals of *R. ferrumequinum* caught using the same technique, as well as on five specimens of *M. emarginatus*. These bats came from a mixed colony of the two species. One of the visits of the colony was made in August 2013, when only a few *M. emarginatus* individuals remained among about a thousand *R. ferrumequinum* bats. However, the bugs were numerous and recently fed. It is therefore clear that *Plecotus* and *Rhinolophus* spp. are able to serve at least as occasional or temporary, though likely less suitable, hosts of cimicids.

Moreover, the preference for a specific host was not detected in host-specificity experiments. *C. pipistrelli* repeatedly sucked on the bat species in whose roosts they have never been observed (Zedníková 2010). In conclusion, the bugs of the genus *Cimex* appear to be common ectoparasites of 20 bat species in Europe (Table 1). The difference between the ranges of host species of *C. pipistrelli* and *C. lectularius* may suggest different host preferences. These preferences can result from different ecologies of the respective bat species (tree or building dwellings versus large attics), as *C. lectularius* is found mostly on attic-dwelling bat species. However, the historical distribution of some of the host bat species, especially *Myotis myotis*, may have played a more important part.

Geographic distribution of cimicids and their hosts

While *Cimex lectularius* as a parasite on man is cosmopolitan, the distribution of the bat-related lineage has never been reviewed. To our knowledge, the records come from the following countries: Afghanistan (Usinger 1966), the Czech Republic (Povolný 1957), Finland (Poppius 1912), France (Usinger & Beaucournu 1967), Germany (e.g. Eichler 1937), Serbia (Protić & Paunović 2006, misidentified as *C. pipistrelli*) and Slovakia (Usinger 1966). Our records extend the known distribution to Hungary, Switzerland (Table 1) and Ukraine (Table 2 in Balvín et al. 2012b, record from a mist-netted bat).

The distribution of *Cimex pipistrelli* has been recently reviewed by Péricart (1996). More recent records of *C. pipistrelli* are by Krištofík & Kaňuch (2006, Slovakia) and Simov et al. (2006, Bulgaria, Greece). The species was newly recorded from Lebanon, Ukraine and Spain; however, all these findings come from mist-netted bats and were listed already by Balvín et al. (2012b).

As cimicids are parasites of bats in their summer roosts, the geographic distribution of cimicids follows the breeding areas of their host species. Records from overwintering bats are singular (Simov et al. 2006). The host range recorded for *C. pipistrelli* and *C. lectularius* (Table 1), comprising many bat species with diverse ecologies, may suggest that their distribution evenly covers all Europe. However, this is not true based on comprehensive data available from five countries covering a Northwest-Southeast transect across Europe: the Czech and Slovak Republics, Hungary, Serbia and Bulgaria. Although the incidence of cimicids in roosts of crevice-dwelling bat species in central Europe cannot be exactly determined, considering the numerous records in our material or the literature, cimicids can be regarded as more or less frequent in their roosts. The incidence of cimicids in roosts of attic-dwelling bats in Central Europe appears to be high.

This situation is in contrast with the frequency of records from Bulgaria. Records from 1997-2006, coming from the comprehensive survey of about 500 roosts in caves, buildings, bat-boxes and tree holes, as well as from examination of about 20000 captured bats were summarized by Simov et al. (2006). They comprise the finding of the newly described *C. emarginatus* in a roost of *M. emarginatus* and only two records of *C. pipistrelli* from *Nyctalus noctula*. Since 2011, an even more extensive survey as part of Natura 2000 has covered about 1600 bat roosts. Furthermore, an additional ca. 10000 mist-netted bats were examined for ectoparasites during the period 2007-2013 in the Tabachka Bat Research Station. During these surveys, no cimicids were found. In addition to these surveys, 1) the second record of *C. emarginatus* was made; 2) *C. pipistrelli* was found in Primorsko, as mentioned above; 3) *C. pipistrelli* was found in 2004 (though only at a photograph) and in 2013 in a bat-box inhabited by *Nyctalus noctula* at Sedemte Prestola Monastery (Western Balkan Mts., see Supplementary material Table 2); and 4) unidentifiable *Cimex* specimens were found in 2006 in guano under a roost likely inhabited by *Nyctalus noctula* in the entrance of Devetashka cave (Lovech Province, see Supplementary material Table 2).

Numerous roosts of *Myotis emarginatus* were examined in southern Vojvodina, a northern province of Serbia, documenting the likely reason for the absence of *C. lectularius* in bat roosts in the southern Balkans. If the non-dwelling bat species *M. myotis* and *M. emarginatus* can be regarded as the principal hosts for *C. lectularius*, as suggested by the limited records from other bats, the absence of *C. lectularius* on bats in the Balkans can be explained by the characteristics of roosts, as suggested by Simov et al. (2006). The temperature in deep caves, where *Myotis* spp. typically roost, is usually below 15 °C, while the humidity often reaches 80-90 % (e.g. Paksuz et al. 2007). The combination of low temperature and high humidity has been shown to be unfavourable or even lethal for *C. lectularius*, and its development is arrested below 13-15 °C (Kemper 1936, Omori 1941). In central Europe, these bat species are synanthropic, inhabiting warm and dry attics. In more southern areas, they mostly occupy their natural habitats of large caves, which are often too humid and cold for cimicids. In Vojvodina province, northern Serbia, *C. lectularius* seems to be common in roosts of *M. emarginatus* in

buildings, whereas in the roosts in caves in central and south Serbia, cimicids have not been recorded.

However, the reason for such low frequency of records of *C. pipistrelli* in Bulgaria and Serbia is less clear. Only about one third of the bat roosts examined during the Natura 2000 mapping in Bulgaria were in humid and cold caves, which are often inhabited by *M. myotis* and *M. emarginatus*. The rest inhabited shallow, dry caves or their entrances (e.g. the entrance of Devetashka cave, Bulgaria, where remains of bugs were found), tree holes, bat-boxes and buildings which should be suitable for cimicids.

As an explanation for the low frequency of records of both *Cimex* species in southern Europe, it is possible that stable colonies of attic-dwelling bats like *M. myotis* provide long-term local reservoirs for infestations in roosts of crevice-dwelling bats. Colonies of such bat species as *Pipistrellus* spp. or *Nyctalus* spp. often split and change roosts during the breeding season, a phenomenon that has been described as the fission-fusion behavioural model (Kummer 1971). This behaviour efficiently reduces the numbers of bugs in the roosts and may be occurring, at least partially, for this purpose (Bartonička & Růžičková 2012). Infestations of a local population of a bat species can eventually be eliminated through this behaviour. This is supported by genetic data on both *C. lectularius* (Balvín et al. 2012a, Booth et al. 2015) and *C. pipistrelli* (Balvín et al. 2013, Wawrocka in litt.), which show no host-associated structure and suggest frequent switching between bat species within regions. In southern Europe, stable infestations in roosts of attic-dwelling bats are absent and therefore cannot be the source of re-infestation of crevice-dwelling bats.

However, this is only partly true for *Nyctalus* species, at least *N. noctula*. Almost all records of *C. pipistrelli* from southern Europe [Bulgaria, Greece (Simov et al. 2006) and Italy (Lanza 1999)] and Lebanon (Balvín et al. 2012b) are from *N. noctula*. Only the record from Spain (Balvín et al. 2012b) was from *N. lasiopterus*. The southern limit of the breeding area of *N. noctula* is 48° N (Kaňuch & Celuch 2004). In summer, only males and non-reproductive females are found south of this limit. As suggested by Simov et al. (2006), it is possible that all the records of *C. pipistrelli* from southern Europe are only temporary transmissions by *N. noctula*. However, at least in the bat-boxes in Cherven and Sedemte Prestola Monastery (Bulgaria), *C. pipistrelli* was found in two subsequent years (Simov et al. 2006, Supplementary material Table 2). It is therefore possible that stable populations may occur here. *N. noctula* transmits cimicids much more often (Balvín et al. 2012b) and is much more migratory than other bat species. Therefore, if the hypothesis of local reservoirs of cimicids in *M. myotis* roosts is valid, in situation when the reservoirs are missing, the local populations in *N. noctula* roosts are not likely to die off like in other crevice-dwelling bat species.

In conclusion, the distribution of *Cimex lectularius* and *C. pipistrelli* is not consistent across the areas of distribution of their host bat species (i.e. different host relations are found in different areas). The distribution of cimicids is presumably shaped by the ecology of bat species. However, it is possible that some bat species provide reservoirs of cimicids, and changes in their ecology across their area of distribution may affect populations of cimicids on other bat species.

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Supplementary online materials

Table 2. List of records of cimicids in bat roosts. IC – identification code of samples in the collection of Ondřej Balvín. Unlabeled collections are deposited in the collection of Tomáš Bartonička; CC – country code (BG – Bulgaria, CZ – Czech Republic, FI – Finland, FR – France, GE – Germany, HU – Hungary, CH – Switzerland, RS – Serbia, SK – Slovakia, UK – United Kingdom); HS – host species: more species listed mean mixed colonies (UBS – unknown bat species, *Eser – Eptesicus serotinus*, *Mbra – Myotis brandti*, *Mdau – M. daubentonii*, *Mema – M. emarginatus*, *Mmyo – M. myotis*, *Mbly – M. blythii*, *Moxy – M. oxygnathus*, *Mnat – M. naterreri*, *Nnoc – Nyctalus noctula*, *Pip* sp. – *Pipistrellus* sp. (*P. pipistrellus* or *P. pygmaeus*), *Ppyg – Pipistrellus pygmaeus*, *Reur – Rhinolophus euryale*, *Rfer – R. ferrumequinum*; NF – number of female bats at the collony; BS – cimicid species (*Clec – Cimex lectularius*, *Cpip – C. pipistrelli*). (Excel file; URL: http://www.ivb.cz/folia/download/balvin_supplementary_table_2.xls).