Research Article

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Deployment and transcriptional evaluation of nitisinone, an FDA-approved drug, to control bed bugs

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Abstract

BACKGROUND: Bed bugs are blood-feeders that rapidly proliferate into large indoor infestations. Their bites can cause allergies, secondary infections and psychological stress, among other problems. Although several tactics for their management have been used, bed bugs continue to spread worldwide wherever humans reside. This is mainly due to human-mediated transport and their high resistance to several classes of insecticides. New treatment options with novel modes of action are required for their control. In this study, we evaluated the use of nitisinone (NTBC), an FDA-approved drug, for bed bug control in an insecticide-susceptible (HH) and an insecticide-resistant (CIN) population.

RESULTS: Although NTBC was lethal to both populations when administered orally or applied topically in very low doses, we observed a slight but significant resistance in the CIN population. Transcriptomic analysis in both populations indicated that NTBC treatment elicited a broad suppression of genes associated with RNA post-transcriptional modifications, translation, endomembrane system, protein post-translational modifications and protein folding. The CIN population exhibited higher adenosine triphosphate (ATP) production and xenobiotic detoxification. Feeding studies on a mouse model suggest that NTBC could be used as a control method of bed bugs by host treatment.

CONCLUSION: The results indicate that NTBC can be used as a new active ingredient for bed bug control by topical or oral treatment and shed light on the molecular mechanisms of suppressed tyrosine metabolism following NTBC treatment.

Supporting information may be found in the online version of this article.

Keywords: hematophagous vector control; *Cimex lectularius*; tyrosine catabolism; 4-hydroxyphenylpyruvate dioxygenase; ectocide

1 INTRODUCTION

The common bed bug, *Cimex lectularius* (Hemiptera: Cimicidae), is an indoor pest that poses serious global public health concerns by infesting not only residential settings but also hotels, cinemas, hospitals and public transport vehicles. Furthermore, bed bugs can be a significant pest of poultry facilities.[1] Over the past two decades, bed bug infestations have resurged globally.[2] Although bed bugs are not known to vector any human pathogens under natural conditions, their bites can cause secondary infections, allergies, psychological stress and other health issues.[3] Bed bug infestations are difficult to eliminate due to their rapid evolution of high resistance to commonly used insecticides such as pyrethroids, neonicotinoids and pyrroles. As a result, multiple treatments with different types of insecticides may be necessary to eradicate an infestation.[4]

Various mechanisms of resistance have been described in bed bugs, including mutations in the target sites that reduce the binding of insecticides,[5] changes in the cuticle that decrease insecticide penetration,[6] and metabolic resistance that enhances the detoxification of insecticides.[7] Specifically, the up-regulation of certain detoxification enzymes like cytochrome P450-dependent

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monooxygenases (P450s), glutathione *S*-transferases (GSTs), carboxylesterases (CESTs), and esterases (ESTs) have been reported to be involved in metabolic resistance in different populations of bed bugs.[7–9] Other mechanisms such as sequestration of the insecticide, the microbiota, oxidative stress and avoidance behavioral adaptations have also been reported to influence insecticide resistance in other insects, such as mosquitoes.[10] Given the widespread resistance of bed bug populations to currently used insecticides and the pervasive cross-resistance to various insecticidal products, new active ingredients with different modes of action are needed to control bed bug populations.

Hematophagous arthropods can consume many times their body weight in a single blood meal, and they have evolved specific adaptations to prevent toxicity from the high quantities of amino acids, heme, iron, and salts generated during vertebrate blood digestion.[11–13] Specifically, it was recently shown that the tyrosine degradation pathway enables blood-feeding insects to tolerate high amounts of free tyrosine produced upon digestion of vertebrate blood proteins.[14] Blocking this pathway causes the accumulation of toxic amounts of tyrosine during blood digestion, ultimately causing the death of blood-fed arthropods without affecting organisms that feed on other diets. This discovery led us to propose that inhibiting tyrosine catabolism might be a novel approach to selectively target hematophagous vectors.[14] Inhibitors of the second enzyme in the tyrosine catabolism pathway, 4-hydroxyphenylpyruvate dioxygenase (HPPD), are commonly used as herbicides and as therapeutic agents in human health.[15] Among the inhibitors tested for vector control, nitisinone (NTBC) was the most effective.[16–18] NTBC is an orphan drug used to treat Tyrosinemia Type I[19] and Alkaptonuria,[20] rare medical conditions that require government intervention to subsidize drug development. When orally administered with the blood meal or topically applied, this drug was lethal to ticks, mosquitoes, tsetse flies, and kissing bugs.[14,16–18,21]

In this study, we assessed the effectiveness of NTBC as a new method for controlling bed bugs in an insecticide-susceptible (Harold Harlan: HH) and an insecticide-resistant (Cincinnati: CIN) population through ingestion and topical application. The death of hematophagous arthropods is due to tyrosine accumulation,[14,18] but the molecular and biochemical pathways affected by this drug are unknown. To gain insight into the effects of NTBC treatment on bed bug biology and the potential mechanisms of resistance to it, we performed a transcriptomic analysis in both populations. Altogether, the results suggest that NTBC could be an effective insecticide for controlling bed bugs by causing a general dysfunction in protein metabolism that can be used as an oral or topical treatment. This approach solely targets hematophagous ectoparasites and disease vectors, making it a more eco-friendly alternative to conventional insecticides.

2 MATERIALS AND METHODS

2.1 Ethics statement

Hairless mice obtained from the Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Brazil, were used for *in vivo* experiments. The animals were housed in standard plastic rodent cages with wood shavings as bedding material. The rodents were maintained on commercial food pellets, and water was provided *ad libitum*. These procedures and protocols were reviewed and approved by the Comissão de Ética no Uso de Animais (CEUA-UFMG 263/2021 – valid up to 27 March 2027).

2.2 Rearing of insects

The HH population was collected in Fort Dix, NJ, USA in 1973 and is also referred to as the Fort Dix strain. They have been kept in plastic containers with cardboard shelters in an environmental chamber at 25 °C, 50 ± 5% relative humidity and a 12-h light/dark cycle at the University of Cincinnati (USA). This population has not been exposed to insecticides since 1973 and is therefore used as a reference strain for susceptibility testing.[7] Similarly, the CIN population was collected in 2012 and reared in the same conditions as HH. Males from the CIN population have recently shown moderate resistance to fipronil and deltamethrin when applied topically.[7] Both populations have been reared under laboratory conditions and fed on defibrinated rabbit blood (Hemostat, Dixon, CA, USA) once a month with an artificial blood feeder (Hemotek, Blackburn, UK).

The bed bug population used in the *in vivo* studies was collected in 2014 and 2015 from public shelters in Belo Horizonte, Brazil (19° 55′ S 43° 56′ W). The insects were kept in a controlled environment at 27 °C, 70% humidity, and 12 h of light/dark cycles at the UFMG since that date, without the introduction of external material or exposure to insecticides.

2.3 Artificial feeding assay

NTBC (PHR1731; Sigma-Aldrich, St Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS) 1× (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM and KH_2PO_4 1.8 mM) immediately before use. The pH was adjusted to 7–8 with 1 M sodium hydroxide (NaOH) and 1 M hydrochloric acid (HCl). To achieve the final concentration for feeding, one volume of NTBC in PBS was mixed with nine volumes of defibrinated rabbit blood (Hemostat). Healthy insects, including first-instar nymphs, adult females and males of unknown age, were fed on rabbit blood mixed with NTBC through an artificial feeding apparatus (Hemotek) at 37 °C. Insects in the control group were fed on rabbit blood containing one volume of PBS 1×. The final NTBC concentrations in blood were 0.05, 0.1, 0.2, 0.3, 1, 3 and 9 μg/mL. The insects were held in the same conditions as described earlier, and their mortality was assessed daily.

2.4 Topical application assay

Adult bed bugs were topically treated with NTBC dissolved in acetone using a 2 μL micropipette (Gilson, Middleton, WI, USA) immediately (0.5–2 h) after feeding on defibrinated rabbit blood. NTBC was dissolved in acetone immediately before use. Each bed bug received 0.5 μL of solution on the ventral thorax/abdomen. The NTBC dosages applied were 750, 250, 83.3, 27.8, 9.3 and 3.1 ng/insect. Bed bugs in the control group received acetone only. The insects were kept in the same conditions as described earlier and the mortality was assessed daily.

2.5 Ecdysis

The daily count of exuviae was used to determine the number of first instar nymphs that molted. Only bed bugs that survived for more than 5 days after ingesting NTBC-supplemented blood were considered for calculating the percentage of successful ecdysis.

2.6 Oviposition and hatching assays

We used females of unknown ages, so all females were assumed to have mated before entering the assays. After undergoing NTBC treatment, both oral and topical treatment, the females were separated into individual vials and kept under the same conditions as earlier. Three weeks after the treatment, the number of eggs laid by each female was counted, along with the number of hatched

nymphs. The ratio of hatching was calculated by dividing the number of hatched first-instar nymphs by the number of eggs laid by each female.

2.7 RNA isolation and RNA-sequencing analysis

Thirty-five adult female insects were collected from the HH and CIN populations 24 h after being fed on blood supplemented either with 0.3 μg/mL NTBC or PBS. Only live healthy insects were collected, and five insects were pooled in a 1.5 mL Eppendorf tube containing 1 mL of cooled TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Seven samples were collected for each condition. The insects were homogenized using BeadBlaster 24 microtube homogenizers (Benchmark Scientific, Sayreville, NJ, USA). RNA from the mixture was separated according to the TRIzol manufacturer's protocol. Afterwards, DNA impurities were eliminated using DNase I (Thermo Fisher Scientific, Waltham, MA, USA). The total RNA was then concentrated using the GeneJET RNA Cleanup and Concentration Micro Kit (Thermo Fisher Scientific). The concentration and quality of the RNA were checked using a Nanodrop spectrophotometer (Thermo Fisher Scientific). The samples were then sent in dry ice for sequencing to Novogene (Sacramento, CA, USA). The three samples that showed better RNA integrity number (RIN) values for each condition (HH-PBS, HH-NTBC, CIN-PBS and CIN-NTBC) were chosen for sequencing. The raw RNA-sequencing (RNA-Seq) data was uploaded to the National Center for Biotechnology Information (NCBI) Sequence Read Archive, Bioproject PRJNA1119180.

2.8 Quality assessment and analysis of RNA-Seq datasets

RNA-Seq analyses were performed as previously described.[22,23] Raw reads were trimmed using Trimmomatic (version 0.38)[24] and the quality of reads was assessed with FastQC (version 0.11.5. https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trimmed sequences were mapped to the bed bug genome GCA_000648675.3[25] using HISAT2 (version 2.1.0).[26] Gene count tables were generated with HTseq (https://htseq.readthedocs.io/en/latest/) under default settings. Differentially expressed genes were determined using the generalized negative binominal model implemented in DESeq2 (version 1.18.1) in R (R Core Team, version 3.5.2). The *P* values were corrected for multiple testing using the false discovery rate (FDR) approach and considered differentially expressed if the *P* value was smaller than or equal to 0.05. The analyses were conducted to compare the effect of NTBC treatment in the two populations (CIN and HH) and a nested analysis was conducted to establish more general effects of NTBC treatment on bed bug biology for both HH and CIN populations. After the identification of the contigs with differential expression, enriched functional groups were identified with g:Profiler[27] and clustered with REVIGO.[28] The differentially expressed genes were searched against two reference protein databases (*Drosophila melanogaster* and SwissProt database) with the use of tBlastx. Functional annotations were assigned to each gene based on this comparison. The differentially expressed genes were categorized according to their known function or cell location in other insects, particularly *D. melanogaster*.

2.9 *In vivo* feeding assay

Hairless mice were orally administered with different doses of NTBC dissolved in PBS 1× (0.5, 5, and 30 mg/kg body mass) while control mice received PBS 1×. Three replicates were performed for each dose using different mice on different days. The mice were anaesthetized intraperitoneally with 150 mg/kg ketamine and 10 mg/kg xylazine, and ten adult females *C. lectularius* were allowed to feed on each treated mouse. In total, 30 insects were fed for each NTBC dose. The survival of the insects was monitored every 6 or 12 h for 90 h.

2.10 Statistical analysis

For each dose in the artificial feeding and topical application assays, at least two independent experiments were conducted, with each experimental group consisting of 8–25 insects. The data from multiple experiments were combined to create a single graph. Statistical analysis and graph design were performed using Prism 8.0.2 software by GraphPad Software (San Diego, CA, USA). Survival analysis and the lethal time 50 (LT_{50}: median time that it took 50% of insects to die) calculations were performed using Kaplan–Meier curves and Log-rank (Mantel–Cox) test analysis. For LT_{50} calculation, only the independent experiments that reached 50% mortality before they were finished were considered. A one-way analysis of variance (ANOVA, Dunnett's multiple comparison test) was conducted to calculate the mean survival for each dose and compare each mean with the controls. On day 10 after feeding, when the lethal doses reached 100% of mortality, the lethal concentration (oral administration, LC_{50}) or the lethal dose (topical application, LD_{50}) of NTBC that killed 50% of the insects of each population was determined using log-dose probit-mortality analysis in PoloPlus (LeOra Software Company, Petaluma, CA, USA). The toxicity of NTBC to the CIN population was compared to the HH population using a resistance ratio (RR_{50}), which was calculated as CIN LD_{50}/HH LD_{50}. The confidence limits (CLs) of the RR_{50} were also calculated, and if the 95% confidence interval did not include the value of 1.0, then the LD_{50} values were considered statistically different.[29]

To evaluate for significant differences in the ecdysis process, a two-way ANOVA (Dunnett's multiple comparisons test) was conducted to compare the groups fed sublethal doses of NTBC (0.05, 0.1, and 0.2 μg/mL) and the group fed with PBS. To determine if there were any significant differences in the number of eggs laid by the females and their hatching between the NTBC-treated and control groups, a one-way ANOVA (Dunnett's multiple comparisons test) was conducted.

3 RESULTS

3.1 Survival analysis of *C. lectularius* following NTBC oral administration through artificial feeding

Artificial feeding assays were conducted with first-instar nymphs and adult males and females of HH and CIN bed bug populations using defibrinated rabbit blood supplemented with different concentrations of NTBC (ranging from 0.05 to 9 μg/mL). The LC_{50} values (concentration that killed 50% of the insects 10 days after feeding) for nymphs were 0.17 (95% CL = 0.08–0.3) and 0.24 μg/mL (95% CL = 0.16–0.40) for the HH and CIN populations, respectively (Table 1 and Fig. 1(A)–(C)). According to Sierras and Schal,[30] first-instar bed bug nymphs consume approximately 0.5 μL of blood; therefore, the ingested LD_{50} HH and CIN nymphs were around 0.08 ng and 0.12 ng of NTBC, respectively. The estimated RR_{50} was 1.4 (95% CL = 1.01–1.9), indicating a slight but statistically significant resistance in CIN nymphs (Fig. 1(A)–(C)). The LT_{50} for lethal doses (0.3–9 μg/mL), which killed bed bugs within 10 days, was 4 days PBM (post-blood meal; Supporting Information, Fig. S1(A),(B) and Table 1). The administration of NTBC did not affect the ecdysis process and almost all of the nymphs that

Table 1. Lethal time 50 (days) for the different doses in artificial feeding assays

	PBS	0.1 µg/mL	0.2 µg/mL	0.3 µg/mL	1 µg/mL	3 µg/mL	9 µg/mL	LC_{50} 10 days PBM (µg/mL) (95% CI)	RR_{50} (CIN/HH) (95% CI)
N1 HH	40.7	19.8	4.3	5.5	3.8	4.7	3.9	0.17 (0.08–0.30)	1.4 (1.01–1.9)
N1 CIN	45.1	22.1	28.3	4.4	4	3.7	3.4	0.24 (0.16–0.4)	
Female HH	41.7	26.0	9.5	2.5	2.5	3.0	2.5	0.15 (0.13–0.18)	1.5 (1.11–2.02)
Female CIN	47.0	31.2	30.0	2.75	2.0	2.5	3.0	0.22 (0.16-0.32)	
Male HH	44.7	35.0	23.0	6.5	9.0	9.2	6.7	0.41 (0.10–1.60)	1.19 (0.59–2.38)
Male CIN	39.7	32.3	27.0	8.5	8.2	6.7	8.5	0.49 (0.12–1.63)	

Note: N1, first instar nymph; HH, Harold Harlan population; CIN, Cincinnati population; PBS, phosphate-buffered saline; LC_{50}, lethal concentration of nitisinone (NTBC) that killed 50% of the insects; PBM, post-blood meal; CI, confidence interval; RR_{50}, resistance ratio.

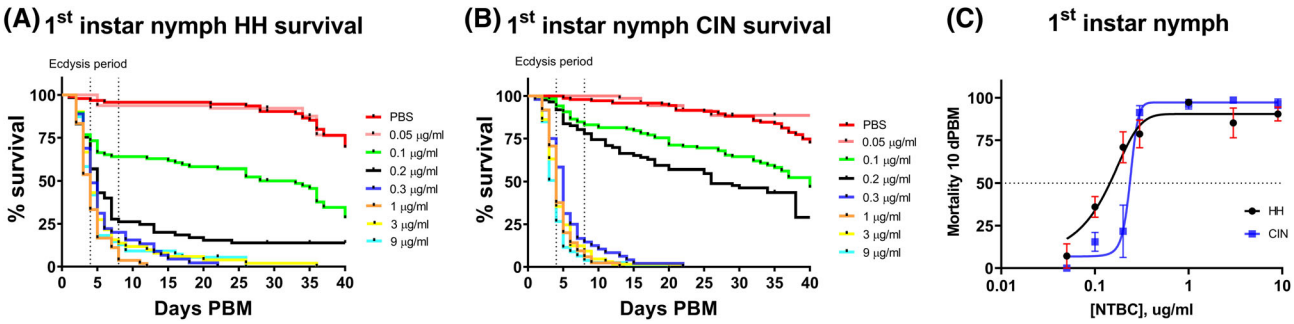

Figure 1. Oral administration of nitisinone (NTBC) is lethal to *Cimex lectularius* nymphs. (A) Survival of the Harold Harlan (HH) and (B) Cincinnati (CIN) populations fed rabbit blood supplemented with NTBC or phosphate-buffered saline (PBS). Three to five independent replicates were performed for each dose. The data from all replicates within a dose were combined for survival analysis and are represented by a single line in each graph. The total number of HH and CIN nymphs used was 513 and 587, respectively. (C) Dose–response curves are shown for HH and CIN nymphs 10 days PBM (post-blood meal), with each point representing mean ± standard error of the mean. All NTBC doses, except 0.05 µg/mL, resulted in higher mortality than controls.

survived longer than 5 days PBM molted to the next instar (Fig. S1(C)).

The oral administration of NTBC to adult insects was also lethal. The LC_{50} for females in the HH and CIN populations was 0.15 (95% CL = 0.13–0.18) and 0.22 µg/mL (95% CL = 0.16–0.32), respectively, with an estimated RR_{50} of 1.5 (95% CI = 1.11–2.02; Fig. 2(A)–(C) and Table 1). Assuming an ingested blood volume of approximately 3.92 µL,[30] the oral NTBC LD_{50} values were 0.59 and 0.86 ng, respectively. The LT_{50} for lethal doses (0.3–9 µg/mL) was 2.6 days (Fig. S2(A),(B) and Table 1), and 100% of mortality was achieved 5 days after feeding with lethal doses. The oral administration of sublethal doses of NTBC did not affect the reproductive fitness of females. There were no differences in the number of eggs laid (Fig. S2(C)) or their hatch rate (Fig. S2(F)) between NTBC-treated females and controls.

The mortality rate of males showed a different pattern. After feeding, it took them a longer time to die, with an LT_{50} of around 8 days for lethal doses (0.3–9 µg/mL. Fig. S2(D),(E) and Table 1), and 100% of mortality was achieved 25 days after feeding on lethal doses (Fig. 2(D),(E)). On day 10 PBM, the LC_{50} values were found to be 0.41 (95% CL = 0.10–1.60) and 0.49 µg/mL (95% CL = 0.12–1.63) for HH and CIN, respectively (Fig. 2(F)). Assuming a blood volume ingested of approximately 3.92 µL,[30] the oral NTBC LD_{50} values were estimated to be 1.6 and 1.9 ng, respectively. The RR_{50} was 1.19 (95% CL = 0.59–2.38), and no significant differences were observed in the susceptibility to NTBC between HH and CIN males when it was orally administered.

3.2 Survival analysis of *C. lectularius* following NTBC topical application

Since most bed bug control strategies nowadays rely on insecticide spray applications that penetrate through the bugs' cuticles, we determined the effectiveness of NTBC through topical applications. Results indicate that the LD_{50} for HH and CIN females was 19.89 (95% CL = 9.76–31.95) and 32.56 ng/female (95% CL = 19.40–46.91), respectively. The RR_{50} was 1.64 (95% CI = 1.01–2.65), similar to that observed for the artificial feeding assays, suggesting that the cuticle might not be involved in resistance towards NTBC in CIN females and pointing to metabolic resistance (Fig. 3(A)–(C) and Table 2). The data indicate that when administered orally to females from both populations, NTBC was 33 and 37 times more potent than when topically applied to HH and CIN females, respectively (Table 3). The LT_{50} for lethal doses (83.3–750 ng/female) was on average 2.7 days (Fig. S3(A),(B) and Table 2). As observed in artificial feeding assays, sublethal doses of NTBC topically applied did not affect the reproductive fitness of females (Fig. S3(C),(F)).

When NTBC was topically applied to males, moderate resistance was observed in the CIN population. The LD_{50} values on day 10 PBM were 14.11 (95% CL = 3.99–30.05) and 61.12 ng/male (95% CL = 32.65–112.19) for HH and CIN, respectively, and the LT_{50} for lethal doses (83.3–750 ng/male) was 4.5 days (Fig. S3(D),(E)). The RR_{50} was 4.3 (95% CL = 2.23–8.30; Fig. 3(D)–(F) and Table 2). This is different from the results obtained in the artificial feeding assays, where no resistance was observed in CIN males

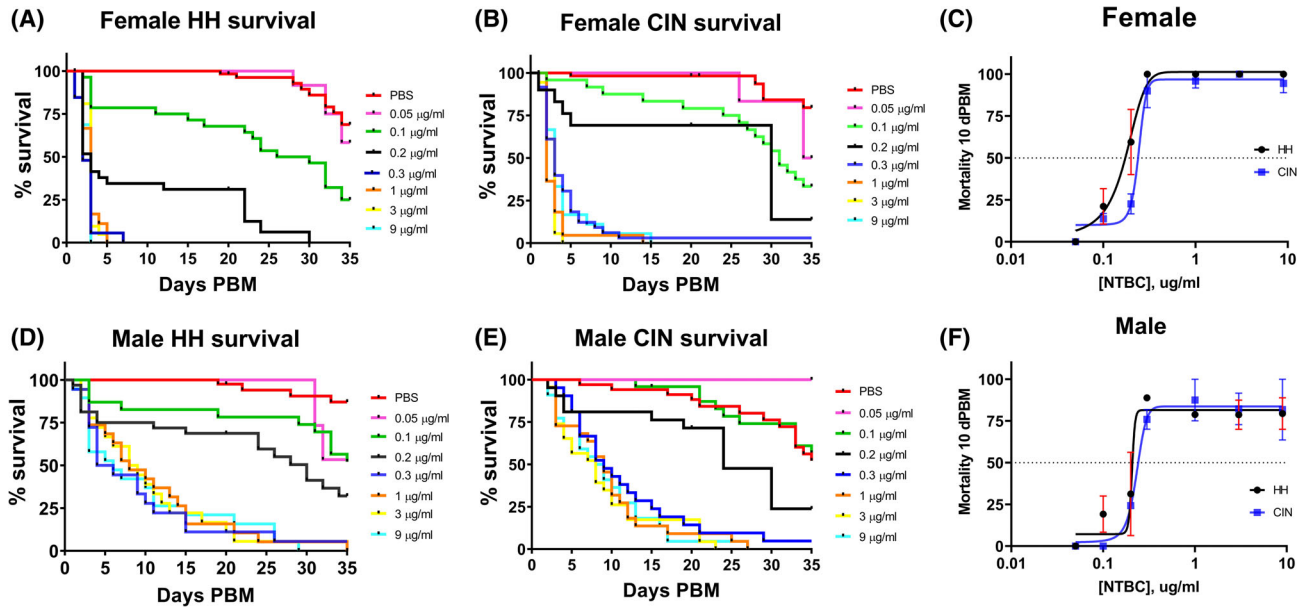

Figure 2. Oral administration of nitisinone (NTBC) is lethal for adult *Cimex lectularius*. Survival of the Harold Harlan (HH) (A) females and (D) males, and Cincinnati (CIN) (B) females and (E) males fed rabbit blood supplemented with NTBC or phosphate-buffered saline (PBS). Two independent replicates were performed for each dose. The data from the two independent replicates for each treatment were combined for survival analysis and represent a single line in each graph. We used 182 HH and 149 CIN females, and 184 HH and 167 CIN males. Dose–response curves are shown for (C) females and (F) males 10 days PBM (post-blood meal), with each point representing mean ± standard error of the mean. All NTBC doses, except 0.05 µg/mL, resulted in higher mortality than controls. In CIN males, the lethality of 0.1 µg/mL was not statically different from controls.

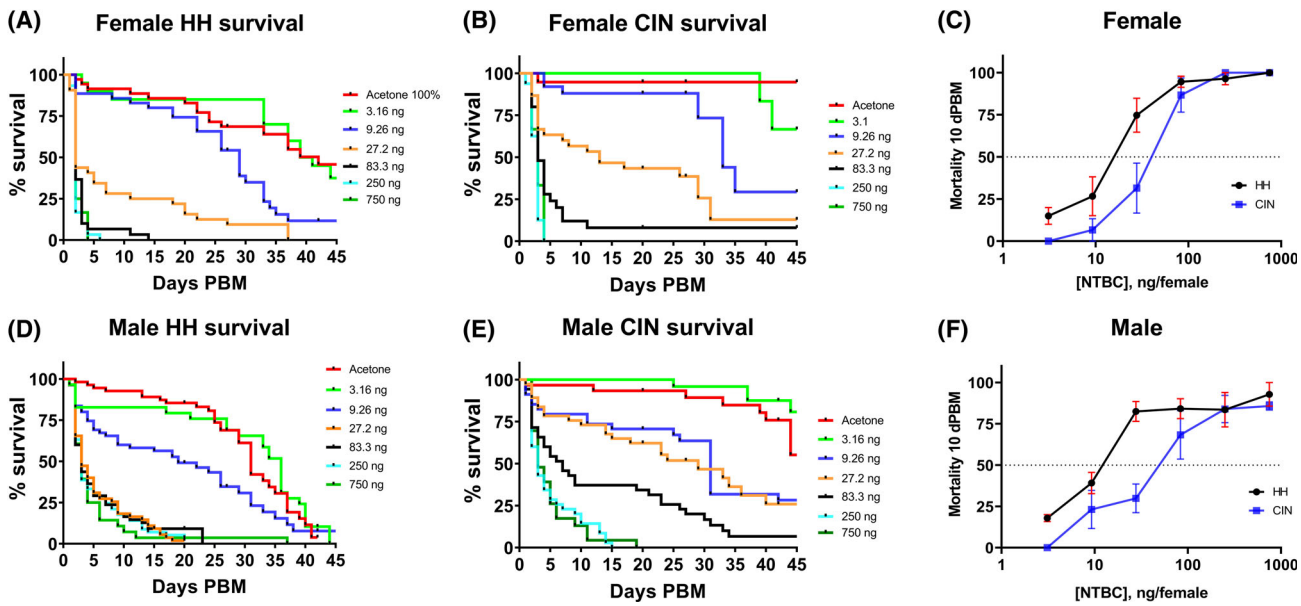

Figure 3. Topical application of nitisinone (NTBC) is lethal for adult *Cimex lectularius*. Survival of the Harold Harlan (HH) (A) females and (D) males, and Cincinnati (CIN) (B) females and (E) males, topically treated with NTBC or acetone as control (0.5 µL/insect). Two to four independent replicates were performed for the different doses. The data from the independent replicates for each treatment were combined for survival analysis and represent a single line in each graph. We used 244 HH and 124 CIN females and 334 HH and 221 CIN males. Dose–response curves are shown for (C) females and (F) males 10 days PBM (post-blood meal), with each point representing mean ± standard error of the mean. All NTBC doses, except 3.1 ng/insect, resulted in higher mortality than controls.

and the LT_{50} was 8 days. However, a delay in the rate of death of males compared to females was observed, as with artificial feeding assays. These findings indicate that the cuticle plays a role in the resistance of CIN males to NTBC. When given orally, NTBC was 9 and 32 times more potent for HH and CIN males, respectively (Table 3).

3.3 Transcriptomic analysis on HH and CIN females fed NTBC

3.3.1 General description of transcriptional changes

The molecular pathways and mechanisms leading to death of blood-feeding arthropods due to tyrosine accumulation after NTBC treatment are still unknown. Besides, it is important to

Table 2. Lethal time 50 (days) for the different doses in topical application assays

	Acetone 100%	3.1 ng	9.26 ng	27.2 ng	83.3 ng	250 ng	750 ng	LD_{50} 10 days PBM (ng)	RR_{50} (CIN/HH) (95% CI)
Female HH	43.0	42.0	22.25	3.5	2.37	2.25	2.0	19.89 (9.76–31.95)	1.64 (1.01–2.65)
Female CIN	>50	>50	34.0	16.5	4.17	2.5	3.0	32.56 (19.40–46.91)	
Male HH	32.5	33.0	17.17	4.37	4.37	4.25	3.0	14.11 (3.99–30.05)	4.33 (2.23–8.30)
Male CIN	47.5	>50	24.67	17.0	9.1	3.1	3.5	61.12 (32.65–112.19)	

Note: HH, Harold Harlan population; CIN, Cincinnati population; LD_{50}, lethal dose of nitisinone (NTBC) that killed 50% of the insects; PBM, post-blood meal; CI, confidence interval; RR_{50}, resistance ratio.

Table 3. LC_{50} [lethal concentration of nitisinone (NTBC) that killed 50% of the insects] and LD_{50} (lethal dose of NTBC that killed 50% of the insects) for artificial feeding assays calculated based on the volume of blood ingested[30] and the LD_{50} calculated on the topical application assays. The last column shows the ratio between lethal dose topical/lethal dose oral

	Artificial feeding LC_{50} 10 days PBM (µL/mL)	Artificial feeding LD_{50} 10 days PBM (ng/insects)	Topical application LD_{50} 10 days PBM (ng/insect)	Lethal dose topical/lethal dose oral
N1 HH	0.17	0.08	—	
N1 CIN	0.24	0.12	—	
Female HH	0.15	0.59	19.9	33.8
Female CIN	0.22	0.86	32.6	37.9
Male HH	0.41	1.61	14.1	8.8
Male CIN	0.49	1.92	61.1	31.8

Note: N1, first instar nymph; HH, Harold Harlan population; CIN, Cincinnati population; PBM, post-blood meal.

understand the mechanisms that might provide resistance to NTBC. To gain insight into how bed bugs respond to NTBC treatments, we conducted a transcriptomic analysis on HH and CIN females fed on blood supplemented with PBS only (control group) or NTBC in PBS that resulted in 0.3 µg/mL blood (the lower lethal dose for both populations). We analyzed the transcript log_2(fold change) values 24 h after feeding. The analysis revealed significant differential expression of many transcripts; 86 and 61 genes were down-regulated in HH and CIN females, respectively; 19 of them were down-regulated in both populations. The up-regulated genes were 50 and 55 in HH and CIN respectively; eight of them were up-regulated in both populations (Fig. S4). Gene ontology (GO) term analysis showed that the down-regulated transcripts are associated with the general processes of protein metabolism, which includes protein folding (GO: 0051082), translational initiation (GO: 0006413), and non-coding RNA (ncRNA) metabolic processes (GO: 0034660) (Fig. 4). The transcripts up-regulated in NTBC-treated bed bugs resulted in enriched GO categories for the HH population that included factors associated with aspartic-type peptidase activity (GO: 0004190). In contrast, the major enriched categories up-regulated in the CIN population include adenosine triphosphate (ATP) synthesis (Fig. 4). A nested analysis with both populations indicates that the nucleoside triphosphate biosynthetic process (GO: 0009141) is generally increased in both populations (Fig. 4).

3.3.2 *Targeted description of transcriptional changes associated with gene groups of interest*

This analysis revealed changes in messenger RNA (mRNA) levels of genes that encode proteins regulating RNA polymerase II transcriptional activity in both populations (Supporting Information, Table S1), suggesting that the activity of this polymerase may be altered. The analysis also revealed the down-regulation of many transcripts encoding proteins associated with RNAs [mRNA, ribosomal RNA (rRNA) and transfer RNA (tRNA)] post-transcriptional modifications and ribosome biogenesis (Table S2). In addition, many subunits of different eukaryotic translation initiation factors (eIF2, eIF3 and eIF4) were down-regulated (Table S3). Moreover, many transcripts coding proteins that play a role in the endomembrane system were also down-regulated in both populations, including proteins involved in protein transport and post-translational modifications (Table S4). Additionally, numerous chaperones and proteasomal proteins were down-regulated (Tables S5 and S6). Peptidase activity was also altered (Table S7).

We also observed that the mRNA levels of many transmembrane transporters (organic and inorganic) were altered. Many of them are mainly expressed in the central nervous system, suggesting that NTBC treatment and/or tyrosine accumulation may impact neural functions (Table S8). A down-regulation in sterol transporters (Niemann–Pick type protein-2 (NPC-2) and NPC-1B) was also observed in both populations (Table S9).

The mRNA levels of genes encoding enzymes for various metabolic pathways were changed. ATP-citrate synthase and glutamine synthetase were down-regulated in both populations (Table S10). Interestingly, tyrosine aminotransferase (TAT), the first enzyme in the tyrosine catabolism pathway, was among the genes that were more up-regulated in both populations. This indicates that the bed bugs respond to tyrosine accumulation due to the inhibition of HPPD by increasing TAT transcription. Additionally, 4-coumarate-CoA ligase, an enzyme involved in the phenylpropanoid biosynthetic pathway, was up-regulated in both populations (Table S10).

The ATP synthase subunits beta, gamma, delta, and epsilon, along with other proteins related to mitochondria function, were up-regulated exclusively in the insecticide-resistant CIN

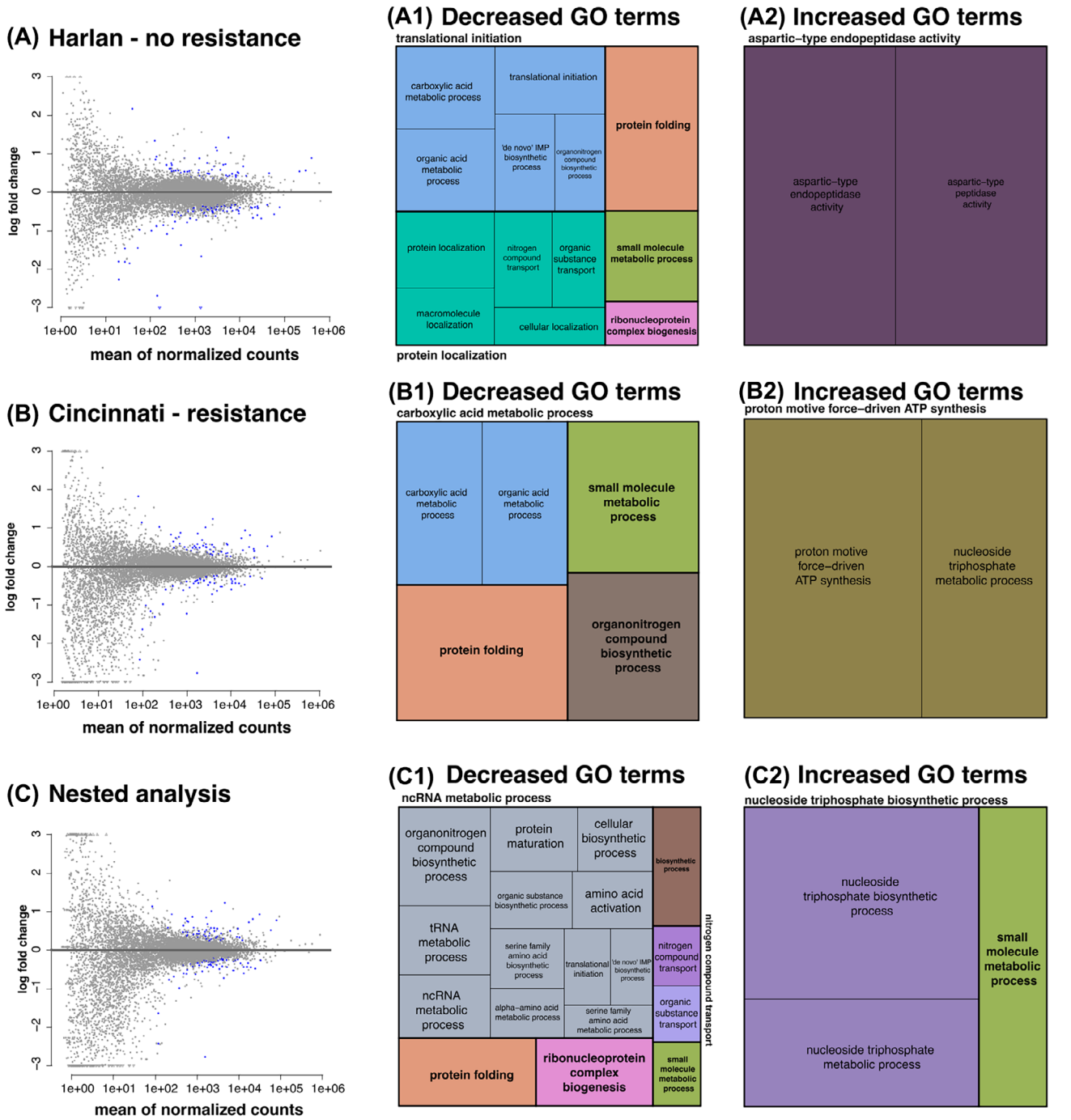

Figure 4. Dispersion plots and gene ontologies (GOs) of interest and their relevant molecular functions, cellular components, and biological processes were visualized using treemaps in nitisinone (NTBC)-treated (A) Harold Harlan (HH) and (B) Cincinnati (CIN) female populations. (C) Nested analyses considering both populations. Blue dots are genes with significant differential expression (FDR, $P < 0.05$). The boxes in the treemaps represent the unique functional categories while the colors represent the major GO groups. The treemaps were generated using REVIGO.

population. Two peroxiredoxins, which play a role in antioxidant function in the mitochondria, were also up-regulated (Table S11). Furthermore, four genes coding for enzymes associated with xenobiotic detoxification were up-regulated only in this population. These enzymes include two GSTs (sigma-1 and theta-1), a CEST, and a cytochrome P450 9e2 (Table S12).

Finally, many uncharacterized genes presented altered mRNA levels upon NTBC treatment. The most down-regulated gene in both populations is uncharacterized (LOC106661977), and its function has not been described in any organism. A leucine-rich repeat-containing protein 4 (LOC106665058) was also down-regulated in both populations. Besides, four up-regulated genes in both populations are uncharacterized (LOC106674390, LOC106674306, LOC106661024, LOC106670295), two of them are described as 'probable salivary secreted peptide' (Table S13).

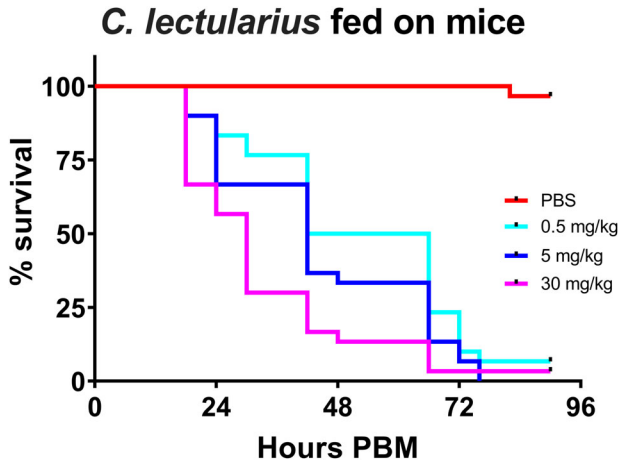

Figure 5. Feeding on mice treated with an oral dose of nitisinone (NTBC) is lethal for *Cimex lectularius*. Three mice were treated with each dose (12 animals in total) and ten adult female bed bugs were fed on each mouse. The total number of bed bugs used was 120. Data are shown as Kaplan–Meier curves. All doses resulted in higher mortality than controls. This experiment used insects obtained from a *C. lectularius* colony established in the Universidade Federal de Minas Gerais from field-collected bed bugs.

3.4 Direct evaluation of host treatment with NTBC for control of *C. lectularius*

People with Tyrosinemia Type I consume a therapeutic oral dose of 1 mg NTBC/kg/day, which results in an NTBC plasma concentration of 8.2 μg/mL, and has a half-life of 54 h.[31] The results of artificial feeding assays suggested that NTBC could be administered to vertebrate hosts as an ectocide to control bed bugs. To test this hypothesis, we conducted *in vivo* experiments. Hairless mice were orally administered with NTBC in three doses: 0.5, 5 and 30 mg/kg, and *C. lectularius* females were fed on them 2–3 h after drug administration. All doses resulted in lethality, presenting an LT_{50} of 2 days PBM (Fig. 5), as observed in the artificial feeding assays. This provides direct evidence that NTBC can be used as an oral treatment for bed bug control that could have applicability in household or agriculture settings.

4 DISCUSSION

The primary method for mitigating indoor bed bug infestations involves using insecticides. However, due to the global increase in bed bug populations and their resistance to currently used pesticides,[4] new alternatives are urgently needed. Our study indicates that inhibiting tyrosine catabolism with NTBC could be a novel approach for controlling bed bugs. Bed bugs are more susceptible to NTBC compared to other blood-feeding insects such as kissing bugs,[14] mosquitoes,[14] tsetse flies[18] and ticks.[21] The insects die due to the buildup of tyrosine from digested proteins, and the time it takes for them to die depends on how quickly they digest their blood meal.[14] We observed unexpected differences in the response to NTBC based on the sex of the bed bugs. Males showed a higher LT_{50} in both insecticide-susceptible and -resistant populations. This difference could be due to a slower digestion rate in males or other sex-related differences in bed bugs. Further investigation is needed to understand the reasons behind these differences.

A small but statistically significant resistance was estimated for CIN nymphs and females ($RR_{50} = 1.6$) upon oral administration, while no resistance was observed in males. Furthermore, the lethal concentration for males in both populations was approximately twice as high as in females from the respective populations. Since the LC_{50} values were calculated on day 10 after feeding for both males and females, the lower rate of male death increases their lethal concentrations and may mask the small resistance that we observed in females. Indeed, the dose of 0.1 μg/mL caused higher mortality in HH males than in the HH controls, but was not different in CIN males from the CIN controls, indicating a difference between HH and CIN male susceptibility to NTBC despite not being statistically significant.

As observed when NTBC was orally administered to bed bugs, differences related to the insects' sex were also observed upon NTBC topical application, with a delay in mortality in males compared with females. The RR_{50} for females in topical application assays was similar to that calculated when NTBC was orally administered. However, different from artificial feeding assays, resistance was observed in CIN males ($RR_{50} = 4.3$). The potency of NTBC was similar to that reported for fipronil when topically applied to HH and CIN males [LD_{50} values equal to 20.3 and 167 ng, respectively ($RR_{50} = 8.4$)]. The CIN males were also resistant to deltamethrin. In this population, P450s were associated with fipronil resistance.[7] Our data suggest that NTBC resistance in CIN males may be associated with the cuticle since no differences were observed in males when NTBC was orally administered. Nevertheless, given the small RR_{50} values, two important caveats might apply to all our oral and topical assays. Firstly, differences in body mass between the two populations and between females and males, if any, might require LD_{50} and LT_{50} values to be normalized per milligram or gram body mass. Secondly, differences in the amount of blood ingested by the two populations and between females and males might also affect the estimated toxicity values and slightly reduce or increase the RR_{50} values.

The administration of sublethal doses of NTBC did not affect the reproductive fitness of females. Besides, females treated with lethal doses laid some eggs before they died. This fact is important in preventing the fixation of resistant alleles in the population, as it reduces the selective pressure, similar to the idealized late-acting insecticides proposed for malaria control.[32,33] This particular characteristic of NTBC is different from neurotoxic insecticides that kill immediately after application, preventing reproduction.

Although tyrosine accumulation and precipitation have been proposed as the mechanism responsible for hematophagous arthropod death after a blood meal due to HPPD inhibition,[12,18] little is known about the molecular mechanisms affected by NTBC and tyrosine. In this study, we identified several cellular processes affected in HH and CIN populations. The RNA-Seq results revealed down-regulation of transcript levels of several genes associated with RNA post-transcriptional modifications, translation, endomembrane system transport, post-translational protein modifications and folding, and protein degradation. In hematophagous arthropods, an increase in protein synthesis occurs coupled with blood meal digestion,[34] the down-regulation of these processes in NTBC-treated insects may indicate that the increase in protein synthesis that normally occurs after a blood meal does not take place.

The levels of many organic and inorganic membrane transporters, some of which are located in the nervous system, were found to be disturbed in both populations. In humans, mental disorders are observed in the different tyrosinemias.[35] Further evaluation is needed to understand the impact of NTBC and tyrosine accumulation on the nervous system of blood-feeding insects and its association with the lethal phenotype.

Importantly, we identify distinct pathways up-regulated in the insecticide-resistant CIN females upon NTBC ingestion, including mitochondrial ATP production and xenobiotic detoxification. The increased peroxidase mRNA levels observed are likely a response to the increased mitochondrial respiration and the consequent production of hydrogen peroxide (H_2O_2). Oxidative stress plays a role in or is a result of resistance to pyrethroids in anopheline mosquitoes. Changes in the redox state have been observed post-pyrethroid exposure.[10,36,37] Besides, an increase in the expression of genes within the oxidative phosphorylation pathway was observed in two *Anopheles coluzzii* resistant populations compared to the susceptible control, which translated phenotypically through an increased respiratory rate.[36,37] The increased mitochondrial activity observed in the CIN population upon NTBC ingestion would help counter the effects of the drug and may contribute to resistance.

Previously, transcriptomic analysis performed in bed bugs showed that strains resistant to deltamethrin presented up-regulated transcript levels of cytochrome P450 and CEST.[8] Also, a comparison of transcriptomes of pesticide-resistant and pesticide-susceptible bed bugs indicated the up-regulation of transcripts involved in penetration resistance and metabolic resistance.[38] Our transcriptional analysis identified four detoxification genes (two GSTs, one P450 and one CEST) that were up-regulated in CIN females fed on NTBC. This up-regulation was not observed in HH females. These enzymes could be involved in the small but significant resistance observed in the CIN population towards NTBC, and perhaps the possible cross-resistance observed with fipronil and deltamethrin in this population.

The effectiveness of certain insecticides was tested for their potential use in creating a liquid bait for bed bugs. Abamectin, clothianidin, and fipronil caused 100% mortality by day 3, while indoxacarb and its bioactive metabolite DCJW were ineffective. Fipronil was the most potent, presenting an LC_{50} in HH adult males equal to 13.4 ng/mL blood (0.052 ng/male).[30] The data presented here indicate the potential use of NTBC in bed bug baits. Moreover, *in vivo* experiments demonstrated that doses as low as 0.5 mg/kg resulted in almost 100% bed bug mortality 3–4 days after they fed on mice treated with NTBC, confirming that it could be used as an ectocide to control bed bugs. The pharmacokinetic profile in humans[31] indicates that the administration of a single therapeutic dose (1 mg/kg) would maintain NTBC blood concentrations above the bed bug LC_{50} (0.25 µg/mL) for around 11 days. Therefore, weekly dosing with NTBC would indefinitely extend its lethal effects on bed bugs. Systemic veterinary drugs are commonly used to control ectoparasites in companion animals and livestock. These drugs have been proposed for ectoparasite control in humans,[39] but with a few exceptions (e.g., ivermectin), their pharmacokinetic properties and potential side effects have not been thoroughly assessed. As bed bugs also proliferate in poultry farms and impact the health and welfare of chickens and workers, systemic drugs like the isoxazoline fluralaner[1,40] and possibly NTBC, have great potential in ectoparasite control by feeding directly to the hosts. These novel treatments could be used individually or combined with other pesticides to be more effective control treatments. Since NTBC has been used in medicine for more than 30 years, it presents the advantage that it could be administered also to humans and likely be administered to a wide range of animals in farm settings due to the minimal side effects.

Collectively, our study provides a foundation for the possible use of NTBC for bed bug control and sheds light on the molecular processes affected by this drug. Moreover, this study may help to elucidate the mechanisms responsible for the low level of NTBC resistance we detected in the CIN population. These mechanisms should be further studied by performing functional assays, such as RNA interference experiments targeting the putative candidate genes and analyzing the susceptibility of bed bugs toward NTBC. This would help devise future strategies to control bed bug populations and manage resistance.

5 CONCLUSIONS

NTBC was highly effective at very low concentrations when given orally and applied topically to bed bugs. The low level of resistance in one field-collected population needs to be assessed with more populations because it falls within the range of variation of such assays. RNA-Seq analyses revealed potential mechanisms of NTBC action, which are likely related to general dysfunction of translation and protein processing. Additionally, oral administration of a low dose of NTBC to a vertebrate host killed nearly 100% of bed bugs colonized from a field collection about a decade ago. These results suggest that NTBC administered orally to vertebrate hosts, including humans, could be a viable method for controlling bed bugs. If used as part of an integrated management strategy, NTBC could improve the elimination of bed bugs in both household and agricultural settings. Furthermore, repurposing NTBC, an FDA-approved drug, for bed bug control may help overcome many regulatory hurdles for both human and animal use.

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DATA AVAILABILITY STATEMENT

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

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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