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

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Abstract 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. 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 ATP production and xenobiotic detoxification. Feeding studies on a mouse model highlight that NTBC could be used as a control method of bed bugs by host treatment. The results demonstrate 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.

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 (González-Morales et al., 2022). Over the past two decades, bed bug infestations have resurged globally (Zorrilla-Vaca et al., 2015). 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 (Akhoundi et al., 2023). 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 (Lee et al., 2023).

Various mechanisms of resistance have been described in bed bugs, including mutations in the target sites that reduce the binding of insecticides (Zhu et al., 2010),

60 changes in the cuticle that decrease insecticide penetration (Lilly et al., 2016), and
61 metabolic resistance that enhances the detoxification of insecticides (González-
62 Morales et al., 2021). Specifically, the upregulation of certain detoxification en-
63 zymes like cytochrome P450-dependent monooxygenases (P450s), glutathione *S*-
64 transferases (GSTs), carboxylesterases (CESTs), and esterases (ESTs) have been re-
65 ported to be involved in metabolic resistance in different populations of bed bugs
66 (Adelman et al., 2011; Bai et al., 2011; González-Morales et al., 2021). Other mech-
67 anisms such as sequestration of the insecticide, the microbiota, oxidative stress and
68 avoidance behavioural adaptations have also been reported to influence insecticide
69 resistance in other insects, such as mosquitoes (Ingham et al., 2023). Given the wide-
70 spread resistance of bed bug populations to currently used insecticides and the per-
71 vasive cross-resistance to various insecticidal products, new active ingredients with
72 different modes of action are needed to control bed bug populations.

73 Hematophagous arthropods can consume many times their body weight in a
74 single blood meal, and they have evolved specific adaptations to prevent toxicity
75 from the high quantities of amino acids, heme, iron, and salts generated during ver-
76 tebrate blood digestion (Graça-Souza et al., 2006; Sterkel et al., 2017; Whiten et al.,
77 2018). Specifically, it was recently shown that the tyrosine degradation pathway en-
78 ables blood-feeding insects to tolerate high amounts of free tyrosine produced upon
79 digestion of vertebrate blood proteins (Sterkel et al., 2016). Blocking this pathway

80 causes the accumulation of toxic amounts of tyrosine during blood digestion, ulti-
81 mately causing the death of blood-fed arthropods without affecting organisms that
82 feed on other diets. This discovery led us to propose that inhibiting tyrosine catabo-
83 lism might be a novel approach to selectively target hematophagous vectors (Sterkel
84 et al., 2016). Inhibitors of the second enzyme in the tyrosine catabolism pathway, 4-
85 hydroxyphenylpyruvate dioxygenase (HPPD), are commonly used as herbicides and
86 in human health (Beaudegnies et al., 2009). Among the inhibitors tested for vector
87 control, nitisinone (NTBC) was the most effective (McComic et al., 2023; Ramirez
88 et al., 2021; Sterkel et al., 2021). NTBC is an orphan drug used to treat Tyrosinemia
89 Type I (Lindstedt et al., 1992) and Alkaptonuria (Ranganath et al., 2016), rare med-
90 ical conditions that require government intervention to subsidize drug development.
91 When orally administered with the blood meal or topically applied, this drug was
92 lethal to ticks, mosquitoes, tsetse flies, and kissing bugs (Cui et al., 2024; McComic
93 et al., 2023; Ramirez et al., 2021; Sterkel et al., 2021, 2016).

94 In this study, we assessed the effectiveness of NTBC as a new method for
95 controlling bed bugs in an insecticide-susceptible (Harold Harlan: HH) and an in-
96 secticide-resistant population (Cincinnati: CIN) through ingestion and topical appli-
97 cation. The death of hematophagous arthropods is due to tyrosine accumulation
98 (Sterkel et al., 2021, 2016), but the molecular and biochemical pathways affected by
99 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) 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 3/27/2027).

2.2. Rearing of insects

The Harold Harlan (HH) population was collected in Ft. Dix, NJ, in 1973 and is also referred to as the Ft. 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-hour 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 (González-Morales et al., 2021). Similarly, the Cincinnati (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 (González-Morales et al., 2021). Both populations have been reared under laboratory conditions and fed on defibrinated rabbit blood (Hemostat, 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 hours of light-dark cycles at the Federal University of Minas Gerais (Brazil) since that date, without the introduction of external material or exposure to insecticides.

2.3. Artificial feeding assay

Nitisinone (PHR1731, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in PBS 1X (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 1M NaOH and 1M HCl. To achieve the final concentration for feeding, one volume of NTBC in PBS was mixed with nine volumes of defibrinated rabbit blood (Hemostat, CA, USA). Healthy insects, including first-instar nymphs, adult females and males of unknown age, were

140 fed on rabbit blood mixed with NTBC through an artificial feeding apparatus
141 (Hemotek) at 37 °C. Insects in the control group were fed on rabbit blood containing
142 one volume of PBS 1X. The final NTBC concentrations in blood were 0.05, 0.1, 0.2,
143 0.3, 1, 3 and 9 µg/ml. The insects were held in the same conditions described above,
144 and their mortality was assessed daily.

145 **2.4. Topical application assay**

146 Blood-fed adult insects received topical applications of NTBC dissolved in ac-
147 etone using a 2 µl micropipette (Gilson) immediately after feeding. NTBC was dis-
148 solved in acetone immediately before use. Each bed bug received 0.5 µl of solution
149 on the ventral thorax. The NTBC dosages applied were 750, 250, 83.3, 27.8, 9.3 and
150 3.1 ng/insect. Bed bugs in the control group received acetone only. The insects were
151 kept in the same conditions described above and the mortality was assessed daily.

152 **2.5. Ecdysis**

153 The daily count of exuviae was used to determine the number of 1st instar
154 nymphs that moulted. Only insects that survived for more than five days after under-
155 going NTBC treatment were considered for calculating the percentage of successful
156 ecdysis.

157 **2.6. Oviposition and hatching assays**

158 We used females of unknown ages, so all females were assumed to have mated

159 before entering the assays. After undergoing NTBC treatment, the females were sep-
160 arated into individual vials and kept under the same conditions as before. Three
161 weeks after the treatment, the number of eggs laid by each female was counted, along
162 with the number of hatched nymphs. The ratio of hatching was calculated by dividing
163 the number of hatched first-instar nymphs by the number of eggs laid by each female.

164 **2.7. RNA isolation and RNA-Seq analysis**

165 Thirty-five adult female insects were collected from the HH and CIN popula-
166 tions 24 hours after being fed on blood supplemented either with 0.3 μg/ml NTBC
167 or PBS. Only live healthy insects were collected, and five insects were pooled in a
168 1.5 ml Eppendorf tube containing 1 ml of cooled TRIzol reagent (Invitrogen, CA,
169 USA). Seven samples were collected for each condition. The insects were homoge-
170 nized using BeadBlaster 24 microtube homogenizers (Benchmark Scientific, NJ,
171 USA). RNA from the mixture was separated according to the TRIzol manufacturer's
172 protocol. Afterwards, DNA impurities were eliminated using DNase I (Thermo Sci-
173 entific, PA, USA). The total RNA was then concentrated using the GeneJET RNA
174 Cleanup and Concentration Micro Kit (Thermo Scientific, PA, USA). The concen-
175 tration and quality of the RNA were checked using a Nanodrop spectrophotometer
176 (Thermo Scientific, MA, USA). The samples were then sent in dry ice for sequencing
177 to Novogene (Sacramento, CA, USA). The three samples that showed better RNA
178 integrity number (RIN) values for each condition (HH-PBS, HH-NTBC, CIN-PBS

179 and CIN-NTBC) were chosen for sequencing. The raw RNA-Seq data was uploaded
180 to the National Center for Biotechnology Information (NCBI) Sequence Read Ar-
181 chive, Bio-project PRJNA1119180.

182 **2.8. Quality assessment and analysis of RNA-Seq datasets**

183 RNA-Seq analyses were performed as previously described (Benoit et al., 2023;
184 Rosendale et al., 2022). Raw reads were trimmed using Trimmomatic (version 0.38)
185 (Bolger et al., 2014) and the quality of reads was assessed with FastQC (version
186 0.11.5. https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trimmed se-
187 quences were mapped to the bed bug genome GCA_000648675.3 (Benoit et al.,
188 2016) using HISAT2 (version 2.1.0)(Kim et al., 2019). Gene count tables were gen-
189 erated with HTseq (https://htseq.readthedocs.io/en/latest/) under default settings.
190 Differentially expressed genes were determined using the generalized negative bi-
191 nominal model implemented in DESeq2 (version 1.18.1) in R (R Core Team, version
192 3.5.2). *P* values were corrected for multiple testing using the false discovery rate
193 (FDR) approach and considered differentially expressed if the *P* value was smaller
194 than or equal to 0.05. The analyses were conducted to compare the effect of NTBC
195 treatment in the two populations (Cincinnati and Harlan) and a nested analysis was
196 conducted to establish more general effects of NTBC treatment on bed bug biology
197 for both HH and CIN populations. After the identification of the contigs with differ-
198 ential expression, enriched functional groups were identified with gProfiler

199 (Raudvere et al., 2019) and clustered with REVIGO (Supek et al., 2011). The differ-
200 entially expressed genes were searched against two reference protein databases
201 (*Drosophila melanogaster* and SwissProt database) with the use of tBlastx. Func-
202 tional annotations were assigned to each gene based on this comparison. The differ-
203 entially expressed genes were categorized according to their known function or cell
204 location in other insects, particularly *Drosophila melanogaster*.

205 **2.9. *In vivo* feeding assay**

206 Hairless mice were orally administered with different doses of NTBC dissolved
207 in PBS 1X (0.5, 5, and 30 mg/kg body mass) while control mice received PBS 1X.
208 Three replicates were performed for each dose using different mice on different days.
209 The mice were anaesthetized intraperitoneally with 150 mg/kg ketamine and
210 10 mg/kg xylazine and ten adult females *C. lectularius* were allowed to feed on each
211 treated mouse. In total, 30 insects were fed for each NTBC dose. The survival of the
212 insects was monitored every 6 or 12 hours for 90 hours.

213 **2.10. Statistical Analysis**

214 For each dose in the artificial feeding and topical application assays, at least
215 two independent experiments were conducted, with each experimental group con-
216 sisting of 8-25 insects. The data from multiple experiments were combined to create
217 a single graph. Statistical analysis and graph design were performed using Prism
218 8.0.2 software by GraphPad Software (San Diego, CA, USA). Survival analysis and

219 the lethal time 50 (LT_{50}: median time that it took 50% of insects to die) calculations
220 were performed using Kaplan-Meier curves and Log-rank (Mantel-Cox) test analy-
221 sis. On day ten after feeding, when the lethal doses reached 100% of mortality, the
222 lethal concentration (Oral administration, LC_{50}) or the lethal dose (Topical applica-
223 tion, LD_{50}) of NTBC that killed 50% of the insects of each population was deter-
224 mined using log-dose probit-mortality analysis in PoloPlus (LeOra Software Com-
225 pany, CA, USA). The toxicity of NTBC to the CIN population was compared to the
226 HH population using a resistance ratio (RR_{50}), which was calculated as CIN
227 LD_{50}/HH LD_{50}. The confidence limits (CLs) of the RR_{50} were also calculated, and if
228 the 95% confidence interval did not include the value of 1.0, then the LD_{50} values
229 were considered statistically different (Wheeler et al., 2007).

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

236

237 # 3. Results

238 ### 3.1. Survival analysis of *C. lectularius* following NTBC oral administration

239 **through artificial feeding**

240 Artificial feeding assays were conducted with first-instar nymphs and adult
241 males and females of HH and CIN bed bug populations using defibrinated rabbit
242 blood supplemented with different concentrations of NTBC (ranging from 0.05 to 9
243 µg/ml). The LC_{50} values (concentration that killed 50% of the insects 10 days after
244 feeding) for nymphs were 0.17 and 0.26 µg/ml for the HH and CIN populations,
245 respectively (Fig. 1A-C). According to (Sierras and Schal, 2017), first-instar bed bug
246 nymphs consume approximately 0.5 µl of blood; therefore, the ingested LD_{50} HH
247 and CIN nymphs were around 0.08 ng and 0.12 ng of NTBC, respectively. The esti-
248 mated RR_{50} was 1.52 (95% CI=1.01-2.11), indicating a slight but statistically signif-
249 icant resistance in CIN nymphs (Fig. 1A-C). The LT_{50} for lethal doses, which killed
250 all bed bugs within 10 days, was on average 4 days PBM (post-blood meal; Fig.
251 S1A-B, Table 1). The administration of NTBC did not affect the ecdysis process and
252 almost all of the nymphs that survived longer than 5 days PBM moulted to the next
253 instar (Fig. S1C).

254 The oral administration of NTBC to adult insects was also lethal. The LC_{50} for
255 females in the HH and CIN populations was 0.15 and 0.25 µg/ml, respectively, with
256 an estimated RR_{50} of 1.64 (95% CI=1.11-2.43; Fig. 2 A-C.). Assuming an ingested
257 blood volume of approximately 3.92 µL (Sierras and Schal, 2017), the oral NTBC
258 LD_{50} values were 0.59 and 0.97 ng, respectively. The LT_{50} was 2.6 days (Fig. S2A-

259 B, Table 1), and 100% of mortality was achieved 5 days after feeding with lethal
260 doses. The oral administration of sublethal doses of NTBC did not affect the repro-
261 ductive fitness of females. There were no differences in the number of eggs laid (Fig.
262 S2C) or their hatch rate (Fig. S2F) between NTBC-treated females and controls.

263 The mortality rate of males showed a different pattern. After feeding, it took
264 them a longer time to die, with an LT_{50} of around 9 days (Fig. S2D-E, Table 1), and
265 100% of mortality was achieved 25 days after feeding on lethal doses (Fig. 2D-E).
266 On day 10 PBM, the LC_{50} values were found to be 0.414 and 0.489 µg/ml for HH
267 and CIN, respectively (Fig. 2F). Assuming a blood volume ingested of approxi-
268 mately 3.92 µL (Sierras and Schal, 2017), the oral NTBC LD_{50} values were estimated
269 to be 1.6 ng and 1.9 ng, respectively. The RR_{50} was 1.18 (with 95% CI=0.51-2.46),
270 and no significant differences were observed in the susceptibility to NTBC between
271 HH and CIN males when it was orally administered.

272 **3.2. Survival analysis of *C. lectularius* following NTBC topical application**

273 Since most bed bug control strategies nowadays rely on insecticide spray ap-
274 plications that penetrate through the bugs' cuticles, we determined the effectiveness
275 of NTBC through topical applications. Results indicate that the LD_{50} for HH and
276 CIN females was 20 and 32.5 ng/female, respectively. The RR_{50} was 1.64 (95%
277 CI=1.01-2.65), similar to that observed for the artificial feeding assays, suggesting
278 that the cuticle might not be involved in resistance towards NTBC in CIN females

279 and pointing to metabolic resistance (Fig. 3A-C, Table 2). The data indicate that
280 when administered orally to females from both populations, NTBC was around 33
281 times more potent than when topically applied (Table 3). The LT_{50} for lethal doses
282 was on average 2.7 days (Fig. S3A-B. Table 2). As observed in artificial feeding
283 assays, sublethal doses of NTBC topically applied did not affect the reproductive
284 fitness of females (Fig. S3C, F).

285 When NTBC was topically applied to males, moderate resistance was observed
286 in the CIN population. The LD_{50} values on day 10 PBM were 14.3 and 61.9 ng/male
287 for HH and CIN, respectively, and the LT_{50} for lethal doses was 4.5 days (Fig S2D-
288 E). The RR_{50} was 4.3 (95% CI=2.23-8.3; Fig. 3D-F, Table 2). This is different from
289 the results obtained in the artificial feeding assays, where no resistance was observed
290 in CIN males and the LT_{50} was 9 days. However, a delay in the rate of death of males
291 in comparison to females was observed, as with artificial feeding assays. These find-
292 ings indicate that the cuticle plays a role in the resistance of CIN males to NTBC.
293 When given orally, NTBC was approximately 9 and 32 times more potent for HH
294 and CIN males, respectively (Table 3).

295 **3.3. Transcriptomic analysis on HH and CIN females fed NTBC**

296 **3.3.1. General description of transcriptional changes**

297 The molecular pathways and mechanisms leading to death of blood-feeding
298 arthropods due to tyrosine accumulation after NTBC treatment are still unknown.

299 Besides, it is important to understand the mechanisms that might provide resistance
300 to NTBC. To gain insight into how bed bugs respond to NTBC treatments, we con-
301 ducted a transcriptomic analysis on HH and CIN females fed blood supplemented
302 with PBS only (control group) or NTBC in PBS that resulted in 0.3 µg/ml blood (the
303 lower lethal dose for both populations). We analyzed the transcript log2 (fold change)
304 values 24 hours after feeding. The analysis revealed significant differential expres-
305 sion of many transcripts; 86 and 61 genes were downregulated in HH and CIN fe-
306 males, respectively; 19 of them were downregulated in both populations. The upreg-
307 ulated genes were 50 and 55 in HH and CIN respectively; 8 of them were upregu-
308 lated in both populations (Fig. S4). GO term analysis showed that the downregulated
309 transcripts are associated with the general processes of protein metabolism, which
310 includes protein folding (GO: 0051082), translational initiation (GO:0006413), and
311 ncRNA metabolic processes (GO:0034660) (Fig. 4). The transcripts upregulated in
312 NTBC-treated bed bugs resulted in enriched GO categories for the HH population
313 that included factors associated with aspartic-type peptidase activity (GO:0004190).
314 In contrast, the major enriched categories upregulated in the CIN population include
315 ATP synthesis (Fig. 4). A nested analysis with both populations indicates that the
316 nucleoside triphosphate biosynthetic process (GO:0009141) is generally increased
317 in both populations (Fig. 4).

318 **3.3.2. Targeted description of transcriptional changes associated with**

319 **gene groups of interest**

320 This analysis revealed changes in mRNA levels of genes that encode proteins
321 regulating RNA polymerase II transcriptional activity in both populations (Table S1),
322 suggesting that the activity of this polymerase may be altered. The analysis also re-
323 vealed the downregulation of many transcripts encoding proteins associated with
324 RNAs (mRNA, rRNA and tRNA) post-transcriptional modifications and ribosome
325 biogenesis (Table S2). In addition, many subunits of different eukaryotic translation
326 initiation factors (eIF2, eIF3 and eIF4) were downregulated (Table S3). Moreover,
327 many transcripts coding proteins that play a role in the endomembrane system were
328 also downregulated in both populations, including proteins involved in protein
329 transport and post-translational modifications (Table S4). Additionally, numerous
330 chaperones and proteasomal proteins were downregulated (Tables S5, S6). Peptidase
331 activity was also altered (Table S7).

332 We also observed that the mRNA levels of many transmembrane transporters
333 (organic and inorganic) were altered. Many of them are mainly expressed in the cen-
334 tral nervous system, suggesting that NTBC treatment and/or tyrosine accumulation
335 may impact neural functions (Table S8). A downregulation in sterol transporters
336 (Niemann-Pick type protein-2 (NPC-2) and NPC-1B) was also observed in both pop-
337 ulations (Table S9).

338 The mRNA levels of genes encoding enzymes for various metabolic pathways

339 were changed. ATP-citrate synthase and glutamine synthetase were downregulated
340 in both populations (Table S10). Interestingly, tyrosine aminotransferase (TAT), the
341 first enzyme in the tyrosine catabolism pathway, was among the genes that were
342 more upregulated in both populations. This indicates that the bed bugs respond to
343 tyrosine accumulation due to the inhibition of HPPD by increasing TAT transcription.
344 Additionally, 4-coumarate-CoA ligase, an enzyme involved in the phenylpropanoid
345 biosynthetic pathway, was upregulated in both populations (Table S10).

346 The ATP synthase subunits beta, gamma, delta, and epsilon, along with other
347 proteins related to mitochondria function, were up-regulated exclusively in the in-
348 secticide-resistant CIN population. Two peroxiredoxins, which play a role in antiox-
349 idant function in the mitochondria, were also upregulated (Table S11). Furthermore,
350 four genes coding for enzymes associated with xenobiotic detoxification were up-
351 regulated only in this population. These enzymes include two glutathione *S*-transfer-
352 ases (Sigma-1 and theta-1), a carboxylesterase, and a cytochrome P450 9e2 (Table
353 S12).

354 Finally, many uncharacterized genes presented altered mRNA levels upon
355 NTBC treatment. The most downregulated gene in both populations is uncharacter-
356 ized (LOC106661977), and its function has not been described in any organism. A
357 leucine-rich repeat-containing protein 4 (LOC106665058) was also downregulated

358 in both populations. Besides, four upregulated genes in both populations are unchar-
359 acterized (LOC106674390, LOC106674306, LOC106661024, LOC106670295),
360 two of them are described as ¨probable salivary secreted peptide¨ (Table S13).
361

362 **3.4. Direct evaluation of host treatment with NTBC for control of *C. lec-***
363 ***tularius***

364 People with Tyrosinemia Type I consume a therapeutic oral dose of 1 mg
365 NTBC/kg/day, which results in an NTBC plasma concentration of 8.2 µg/ml, and
366 has a half-life of 54 hours (Hall et al., 2001). The results of artificial feeding assays
367 suggested that NTBC could be administered to vertebrate hosts as an ectocide to
368 control bed bugs. To test this hypothesis, we conducted *in vivo* experiments. Hairless
369 mice were orally administered with NTBC in three doses: 0.5, 5 and 30 mg/kg, and
370 *C. lectularius* females were fed on them 2-3 hours after drug administration. All
371 doses resulted in lethality, with LT_{50} around 2 days PBM (Fig. 5), similar to the LT_{50}
372 observed in the artificial feeding assays. This provides direct evidence that NTBC
373 can be used as an oral treatment for bed bugs control.
374

375 # 4. Discussion

376 The primary method for mitigating indoor bed bug infestations involves using
377 insecticides. However, due to the global increase in bed bug populations and their

378 resistance to currently used pesticides (Lee et al., 2023), new alternatives are ur-
379 gently needed. Our study indicates that inhibiting tyrosine catabolism with NTBC
380 could be a novel approach for controlling bed bugs. Bed bugs are more susceptible
381 to NTBC compared to other blood-feeding insects such as kissing bugs (Sterkel et
382 al., 2016), mosquitoes (McComic et al., 2023; Sterkel et al., 2016; Vergaray Ramirez
383 et al., 2022), tsetse flies (Sterkel et al., 2021) and ticks (Cui et al., 2024). The insects
384 die due to the buildup of tyrosine from digested proteins, and the time it takes for
385 them to die depends on how quickly they digest their blood meal (Sterkel et al.,
386 2016). We observed unexpected differences in the response to NTBC based on the
387 sex of the bed bugs. Males showed a higher LT_{50} in both insecticide-susceptible and
388 resistant populations. This difference could be due to a slower digestion rate in males
389 or other sex-related differences in bed bugs. Further investigation is needed to un-
390 derstand the reasons behind these differences.

391 A small but statistically significant resistance was estimated for CIN nymphs
392 and females (RR_{50}=1.6) upon oral administration, while no resistance was observed
393 in males. Furthermore, the LC for males in both populations was approximately
394 twice as high as in females from the respective populations. Since the LC_{50}s were
395 calculated on day 10 after feeding for both males and females, the lower rate of male
396 death increases their LCs and may mask the small resistance that we observed in
397 females. Indeed, the dose of 0.1 μg/ml caused higher mortality in HH males than in

398 the HH controls, but was not different in CIN males from the CIN controls, indicat-
399 ing a difference between HH and CIN male susceptibility to NTBC despite not being
400 statistically significant.

401 As observed when NTBC was orally administered to bed bugs, differences
402 related to the insects' sex were also observed upon NTBC topical application, with a
403 delay in mortality in males compared with females. The RR_{50} for females in topical
404 application assays was similar to that calculated when NTBC was orally adminis-
405 tered. However, different from artificial feeding assays, resistance was observed in
406 CIN males (RR_{50}=4.3). The potency of NTBC was similar to that reported for
407 fipronil when topically applied to HH and CIN males (LD_{50} values equal to 20.3 ng
408 and 167 ng, respectively (RR_{50}=8.4)). The CIN males were also resistant to deltame-
409 thrin. In this population, P450s were associated with fipronil resistance (González-
410 Morales et al., 2021). Our data suggest that NTBC resistance in CIN males may be
411 associated with the cuticle since no differences were observed in males when NTBC
412 was orally administered. Nevertheless, given the small RR_{50} values, two important
413 caveats might apply to all our oral and topical assays. First, differences in body mass
414 between the two populations and between females and males, if any, might require
415 LD_{50} and LT_{50} values to be normalized per mg or gram body mass. Secondly, differ-
416 ences in the amount of blood ingested by the two populations and between females

417 and males might also affect the estimated toxicity values and slightly reduce or in-
418 crease the RR_{50} values.

419 The administration of sublethal doses of NTBC did not affect the reproductive
420 fitness of females. Besides, females treated with lethal doses laid some eggs before
421 they died. This fact is important in preventing the fixation of resistant alleles in the
422 population, as it reduces the selective pressure, similar to the idealized late-acting
423 insecticides proposed for malaria control (Koella et al., 2009; Read et al., 2009).
424 This particular characteristic of NTBC is different from neurotoxic insecticides that
425 kill immediately after application, preventing reproduction.

426 Although tyrosine accumulation and precipitation have been proposed as the
427 mechanism responsible for hematophagous arthropod death after a blood meal due
428 to HPPD inhibition (Sterkel et al., 2021, 2016), little is known about the molecular
429 mechanisms affected by NTBC and tyrosine. In this study, we identified several cel-
430 lular processes affected in HH and CIN populations. The RNA-Seq results revealed
431 downregulation of transcript levels of several genes associated with RNA post-tran-
432 scriptional modifications, translation, endomembrane system transport, post-trans-
433 lational protein modifications and folding, and protein degradation. In hematopha-
434 gous arthropods, an increase in protein synthesis occurs coupled with blood meal
435 digestion (Lehane, 2005), the downregulation of these processes in NTBC-treated
436 insects may indicate that the increase in protein synthesis that normally occurs after

437 a blood meal does not take place.

438 The levels of many organic and inorganic membrane transporters, some of
439 which are located in the nervous system, were found to be disturbed in both popula-
440 tions. In humans, mental disorders are observed in the different tyrosinemias (Scott,
441 2006). Further evaluation is needed to understand the impact of NTBC and tyrosine
442 accumulation on the nervous system of blood-feeding insects and its association with
443 the lethal phenotype.

444 Importantly, we identify distinct pathways upregulated in the insecticide-re-
445 sistant CIN females upon NTBC ingestion, including mitochondrial ATP production
446 and xenobiotic detoxification. The increased peroxidase mRNA levels observed are
447 likely a response to the increased mitochondrial respiration and the consequent pro-
448 duction of H_2O_2. Oxidative stress plays a role in or is a result of resistance to pyre-
449 throids in anopheline mosquitoes. Changes in the redox state have been observed
450 post-pyrethroid exposure (Ingham et al., 2021; Ingham et al., 2023). Besides, an in-
451 crease in the expression of genes within the oxidative phosphorylation pathway was
452 observed in two *Anopheles coluzzii* resistant populations compared to the susceptible
453 control, which translated phenotypically through an increased respiratory rate
454 (Ingham et al., 2021). The increased mitochondrial activity observed in the CIN pop-
455 ulation upon NTBC ingestion would help counter the effects of the drug and may
456 contribute to resistance.

457 Previously, transcriptomic analysis performed in bed bugs showed that strains
458 resistant to deltamethrin presented upregulated transcript levels of cytochrome P450
459 and carboxylesterase (Adelman et al., 2011). Also, a comparison of transcriptomes
460 of pesticide-resistant and pesticide-susceptible bed bugs indicated the upregulation
461 of transcripts involved in penetration resistance and metabolic resistance (Mamidala
462 et al., 2012). Our transcriptional analysis identified four detoxification genes (two
463 GSTs, one P450 and one CEST) that were upregulated in CIN females fed on NTBC.
464 This upregulation was not observed in HH females. These enzymes could be in-
465 volved in the small but significant resistance observed in the CIN population towards
466 NTBC, and perhaps the possible cross-resistance observed with fipronil and del-
467 tamethrin in this population.

468 The effectiveness of certain insecticides was tested for their potential use in
469 creating a liquid bait for bed bugs. Abamectin, clothianidin, and fipronil caused 100%
470 mortality by day 3, while indoxacarb and its bioactive metabolite DCJW were inef-
471 fective. Fipronil was the most potent, presenting an LC_{50} in HH adult males equal to
472 13.4 ng/ml blood (0.052 ng/male) (Sierras and Schal, 2017). The data presented here
473 indicate the potential use of NTBC in bed bug baits. Moreover, *in vivo* experiments
474 demonstrated that doses as low as 0.5 mg/kg resulted in almost 100% bed bug mor-
475 tality 3-4 days after they fed on mice treated with NTBC, confirming that it could be
476 used as an ectocide to control bed bugs. The pharmacokinetic profile in humans (Hall

477 et al., 2001) indicates that the administration of a single therapeutic dose (1 mg/kg)
478 would maintain NTBC blood concentrations above the bed bug LC_{50} (0.25 µg/ml)
479 for around 11 days. Therefore, weekly dosing with NTBC would indefinitely extend
480 its lethal effects on bed bugs. Systemic veterinary drugs are commonly used to con-
481 trol ectoparasites in companion animals and livestock. These drugs have been pro-
482 posed for ectoparasite control in humans (Miglianico et al., 2018), but with a few
483 exceptions (e.g., ivermectin), their pharmacokinetic properties and potential side ef-
484 fects have not been thoroughly assessed. As bed bugs also proliferate in poultry
485 farms and impact the health and welfare of chickens and workers, systemic drugs
486 like the isoxazoline fluralaner (González-Morales et al., 2023, 2022), and possibly
487 NTBC, have great potential in ectoparasite control. These novel treatments could be
488 used individually or combined with other pesticides to be more effective control
489 treatments. Since NTBC has been used in medicine for more than 30 years, it pre-
490 sents the advantage that it could be administered also to humans.

491 Collectively, our study provides a foundation for the possible use of NTBC
492 for bed bug control and sheds light on the molecular processes affected by this drug.
493 Moreover, this study may help to elucidate the mechanisms responsible for the low
494 level of NTBC resistance we detected in the CIN population. These mechanisms
495 should be further studied by performing functional assays, such as RNA interference
496 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. Furthermore, repurposing NTBC, an FDA-approved drug, for bed bug control may help overcome many regulatory hurdles.

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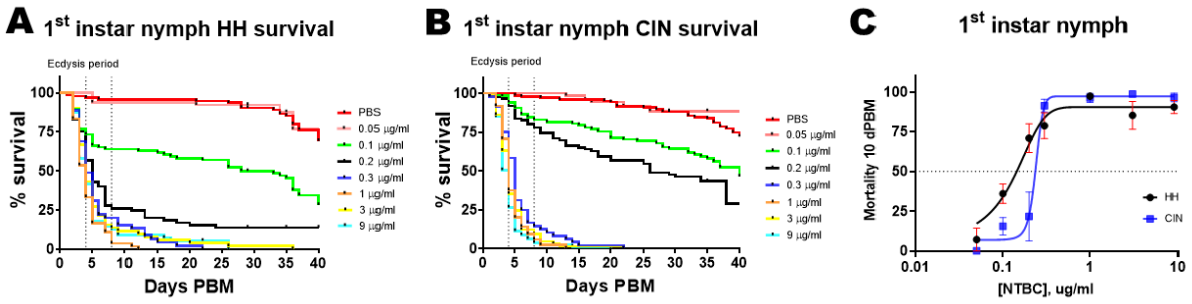
523 # 7. Figure legends



524

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

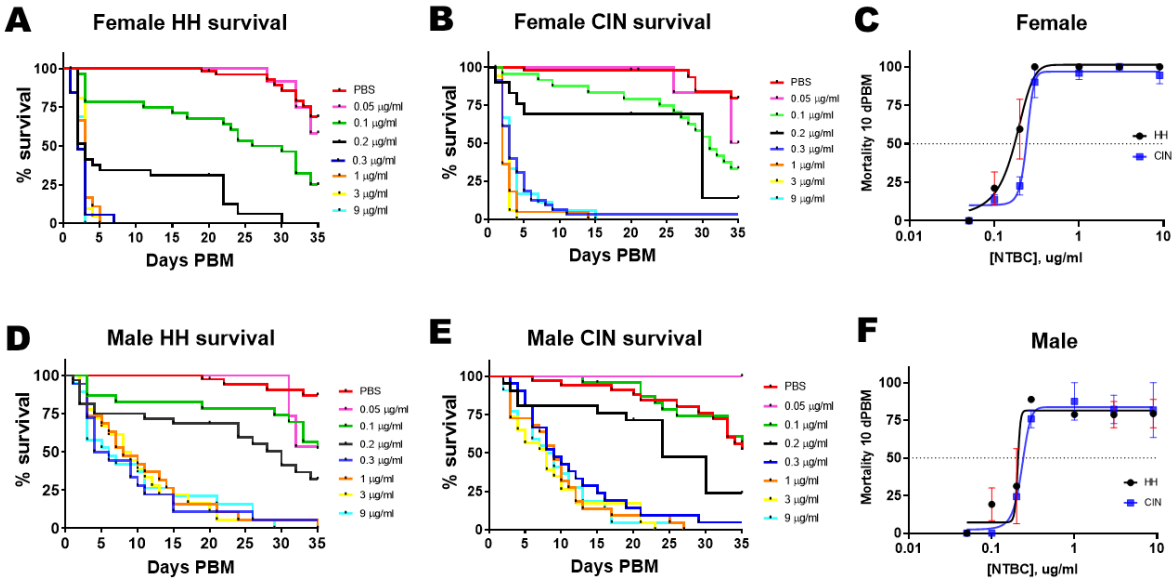


Figure 2: Oral administration of NTBC is lethal for adult *Cimex lectularius*. Survival of the Harold Harlan (HH) **A)** females and **D)** males, and Cincinnati (CIN) **B)** females and **E)** males fed rabbit blood supplemented with NTBC or PBS. Two independent replicates were performed for each dose. 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±SEM. 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.

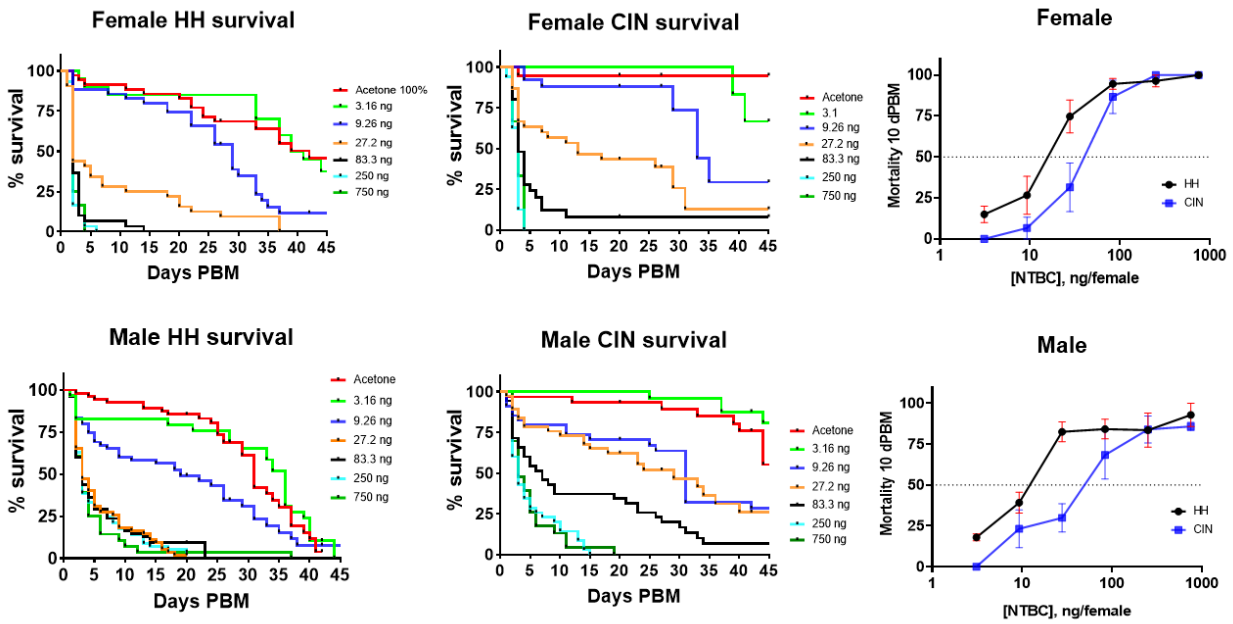


Figure 3. Topical application of NTBC is lethal for adult *Cimex lectularius*. Survival of the Harold Harlan (HH) **A)** females and **D)** males, and Cincinnati (CIN) **B)** females and **E)** males, topically treated with NTBC or acetone as control (0.5 µl/insect). Two to four independent replicates 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±SEM. All NTBC doses, except 3.1 ng/insect, resulted in higher mortality than controls.

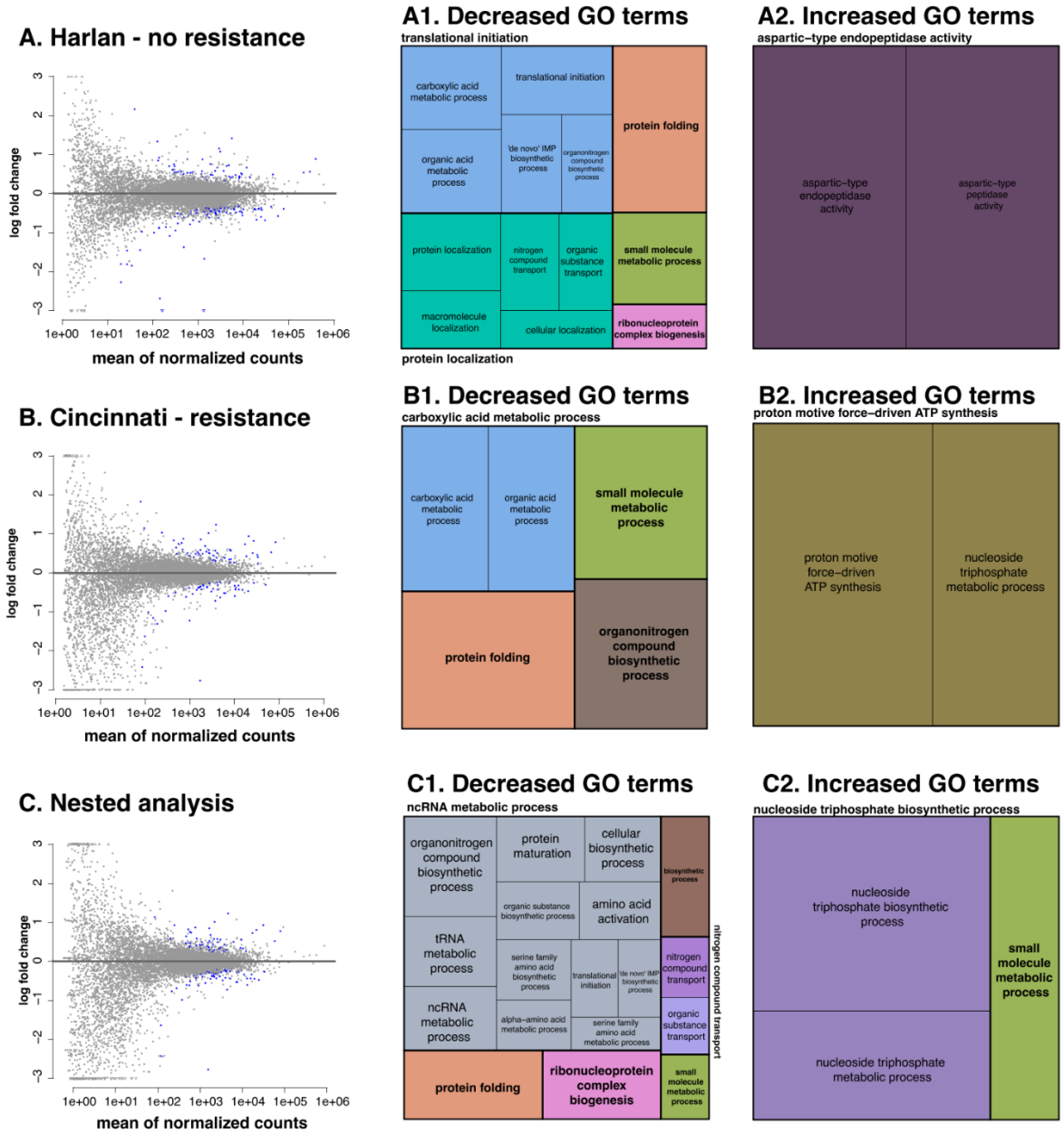


Figure 4. Dispersion plots and gene ontologies (GO) of interest and their relevant molecular functions, cellular components, and biological processes were visualized using treemaps in NTBC-treated **A)** HH and **B)** CIN females. **C)** Nested analyses considering both populations. Blue dots are genes with significant differential expression (FDR, $P < 0.05$). The boxes in the treemaps represent the unique

567 functional categories while the colours represent the major GO groups. The treemaps
568 were generated using REVIGO.
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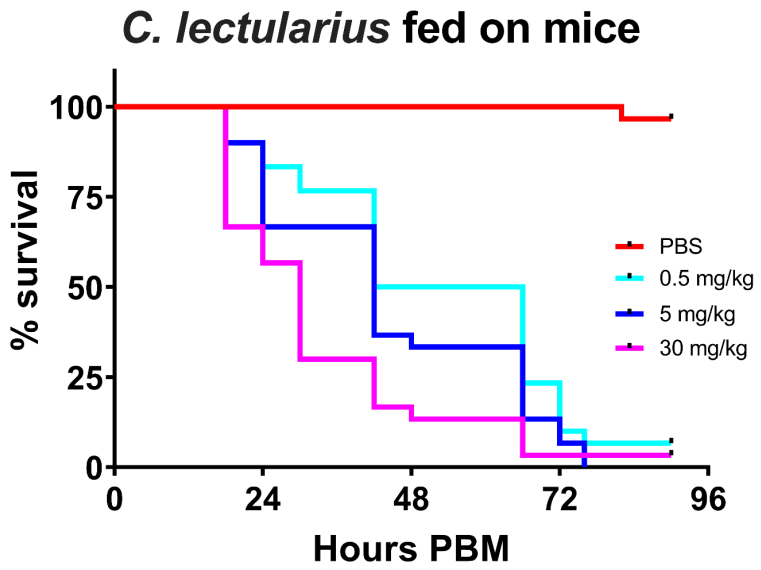

572

573 **Figure 5: Feeding on mice treated with an oral dose of NTBC is lethal for**

574 ***C. lectularius***. Three mice were treated with each dose (twelve animals in total) and

575 10 adult female bed bugs were fed on each mouse. The total number of bed bugs

576 used was 120. Data are shown as Kaplan-Meier curves. All doses resulted in higher

577 mortality than controls. This experiment used insects obtained from a *C. lectularius*

578 colony established in the Universidade Federal de Minas Gerais from field-collected

579 bed bugs.

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583 # 8. Tables

584 **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 dPBM (µg/ml)	RR_{50} (CIN/HH)	95% limits
N1 HH	40.7	19.8	4.33	5.5	3.8	4.75	3.9	0.171	1.52	1.10 to 2.10
N1 CIN	45.1	22.1	23.0	4.4	4.0	3.7	3.4	0.26		
Female HH	41.67	26.0	9.5	2.5	2.5	3.0	2.5	0.151	1.64	1.11 to 2.43
Female CIN	47.0	31.17	30.0	2.75	2.0	2.5	3.0	0.248		
Male HH	44.75	35.0	23.0	6.5	9.0	9.25	6.75	0.414	1.18	0.51 to 2.46
Male CIN	39.75	32.33	27.0	8.5	8.25	6.75	8.5	0.489		

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587 **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 dPBM (ng)	RR_{50} (CIN/HH)	95% limits
Female HH	43.0	42.0	22.25	3.5	2.37	2.25	2.0	19.9	1.64	1.01 to 2.65
Female CIN	>50	>50	34.0	16.5	4.17	2.5	3.0	32.5		
Male HH	32.5	33.0	17.17	4.37	4.37	4.25	3.0	14.3	4.33	2.23 to 8.3
Male CIN	47.5	>50	24.67	17.0	9.1	3.1	3.5	61.9		

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589

590 **Table 3:** LC50 and LD50 for artificial feeding assays calculated based on the volume

591 of blood ingested (Sierras and Schal, 2017), and the LD50 calculated on the topical

592 application assays. The last column shows the ratio between LD topical/LD oral.

	Artificial feeding	Artificial feeding	Topical application	
	LC_{50} 10 dPBM (µl/ml)	LD_{50} 10 dPBM (ng/insects)	LD_{50} 10 dPBM (ng/insect)	LD topical/LD oral
N1 HH	0.17	0.08	-	
N1 CIN	0.26	0.12	-	
Female HH	0.15	0.59	19.9	33.82
Female CIN	0.25	0.98	32.56	33.22
Male HH	0.41	1.61	14.3	8.9
Male CIN	0.49	1.92	61.9	32.23

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