

Common insecticide affects spatial navigation in bats at environmentally-realistic doses

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Abstract

Bats are potentially exposed to pesticides via foraging in croplands. Common pesticides like organophosphates are neurotoxic for vertebrates and even low doses can impair essential processes such as locomotion and cognition. These sublethal effects are usually studied using molecular biomarkers with limited ecological relevance. Behavioral studies, in contrast, represent a more informative yet sensitive approach. Spatial navigation, for example, is an ecologically relevant behavior that is modulated by cellular pathways potentially targeted by neurotoxicants. We evaluated whether bats' ability to memorize and navigate novel spaces was negatively affected by environmental relevant doses of chlorpyrifos, a common organophosphate insecticide. We also tested how the behavioral response correlated with molecular biomarkers. We orally dosed captive big brown bats (*Eptesicus fuscus*) with chlorpyrifos and studied exploratory behavior in two testing arenas. We evaluated similarity of stereotype flight trajectories in a flight tent, and associative memory in a Y-maze. We quantified brain cholinesterase (ChE) activity as a cellular biomarker and employed non-targeted proteomics as molecular biomarkers. Bats exposed to chlorpyrifos were less explorative and made more incorrect choices in the Y-maze, but the consistency of their flight trajectories was unaffected. Exposed bats had 30% lower ChE activity, showed down-regulation of proteins involved in memory (VP37D), learning and sound perception (NOX3). Other important nervous system processes such as synaptic function, plasticity, oxidative stress, and apoptosis were enriched in chlorpyrifos-exposed bats. These results support the sensitivity of behavior as a biomarker of toxicity and the importance of considering other levels of organization to help explain the mechanisms underlying altered behavior due to human activities.

32 Key words

33 Proteomics, neurotoxicity, cholinesterase inhibition, Y maze, organophosphates

34 Introduction

35 Bats provide essential ecosystem services, such as arthropod suppression, and a sustained,
36 thriving population is vital for ecosystem health [1]. Insectivorous bats, for example, are efficient
37 bio-control agents eating large amounts of insects every night, including agricultural pests [2,3].
38 Foraging in croplands, however, makes bats vulnerable to pesticide exposure through
39 consumption of contaminated prey or coming into direct contact with sprayed toxicants while
40 flying and/or grooming [3–5]. In addition to a high risk of exposure, bats present physiological
41 and ecological characteristics such as high metabolism, low fecundity and long lifespan that
42 could make them especially vulnerable to pesticide effects compared to other non-target
43 vertebrates [4]. Similarly, bat characteristic behaviors, such as echolocation and torpor, may be
44 adversely affected by pesticide exposure and could jeopardize individual fitness. Understanding
45 these threats to bat survival is crucial for conservation and will help to elucidate potential causes
46 of observed population declines worldwide.

47 Commonly used pesticides like organophosphates (OPs) are known to be neurotoxic for non-
48 target species including birds [6,7], fish [8,9], and humans [10,11], but little is known about their
49 effects on bats [12,13]. Studies of OP toxicity in mammalian model organisms, like mice and
50 rats, show impairment of cognitive, motor, sensory, and autonomic functions even at low
51 concentrations (less than 1/50th of lethal dose) [14–16]. These effects, however, may not simply
52 be extrapolated to bats because of unique physiological adaptations associated with flight that set
53 them apart from non-volant mammals [2]. Moreover, physiological and behavioral effects are
54 often sublethal and therefore difficult to detect using traditional toxicological assessments.
55 Hence, integrative approaches that include biomarkers at different levels of biological
56 organization may lead to a better understanding of the deleterious effects of toxicants on wildlife.
57 Behavioral biomarkers in particular can be a sensitive and ecologically relevant alternative to
58 evaluate neurotoxic effects and their implications on animal fitness.

59 Navigation by flight is an ecologically-relevant behavior in bats essential to foraging, finding
60 roosts, migration, and sometimes even reproduction [18]. Bats use multiple cognitive processes,

61 including associative learning and spatial memory, to integrate previous experiences and
62 environmental cues (e.g., position, shape, color) to find resources; however, cognitive processes
63 can be impaired by OP pesticides due to their neurotoxic mode of action. A primary target of
64 OPs is the enzyme cholinesterase (ChE) that hydrolyzes acetylcholine, a major neurotransmitter
65 in the peripheral and central nervous systems [13]. Inhibition of ChE activity causes an
66 accumulation of acetylcholine at cholinergic synapses, and causes increased neural activity
67 [6,19]. Cognitive processes like spatial navigation, learning, olfaction, and visual perception are
68 modulated by cholinergic synapses and could therefore be affected by neurotoxicant exposure,
69 leading to functional disruption of cholinergic pathways.

70 Due to its specificity, quantification of ChE activity has become the gold standard biomarker of
71 neurotoxicity for OPs exposure [20–22]. Although ChE inhibition is the primary mode of action
72 of OPs, other processes such as oxidative stress, DNA damage, and cell death have been linked
73 to neurochemical and neurobehavioral effects of OPs (reviewed in Tsai & Lein, 2021). A more
74 comprehensive understanding of such mechanisms and adverse outcomes, however, requires an
75 exhaustive biomarker analysis, which is limited by available knowledge on the pesticide and
76 study species. Broad characterization of the brain proteome could help reveal, with high
77 resolution, new and relevant molecular pathways of OP toxicity [24–26]. Proteomic analyses
78 provide a large-scale overview of protein-level expression and regulation, which facilitates
79 linking mechanistic impacts at the molecular level to impacts at the individual level, and
80 consequently to populations and/or ecosystems [27].

81 To date, few studies have examined the neurotoxic effects of pesticides on bats and most have
82 focused on a single biomarker, ChE activity. Here, we combine cellular and molecular
83 biomarkers of exposure with behavioural biomarkers to evaluate the neurotoxic effects of an
84 environmentally relevant dose of Chlorpyrifos (CPF), a widely used organophosphate
85 pesticide, on the big brown bat (*Eptesicus fuscus*). To confirm neurotoxicity at the molecular
86 level, we quantified ChE inhibition in the bat brain and analyzed changes in the brain proteome
87 to elucidate other molecular and cellular pathways affected by CPF exposure. Based on the
88 known mode of action, we hypothesized that CPF would cause a reduction in brain ChE activity
89 and in turn affect multiple cholinergic-modulated processes, including cognition. Specifically,
90 we predicted that effects at the cellular (ChE) and molecular level (protein profile) would be
91 reflected by neurobehavioral impairment in spatial navigation tasks such as stereotyped

92 navigation patterns and associative memory. Both behaviors are ecologically relevant for bats
93 and their disruption could be detrimental for fitness and survival.

94 Materials and methods

95 Experimental animals and housing

96 We used big brown bats (*Eptesicus fuscus*) from a captive research colony established at
97 McMaster University. Bats were either wild-caught in Southern Ontario or direct descendants of
98 wild-caught individuals. Bats were housed indoors (2.5 x 1.5 x 2.3 m; l x w x h) in a mixed-sex
99 colony where the temperature and lighting varied with ambient conditions, and bats had access to
100 a larger outdoor flying area (2.5 x 3.8 x 2.7 m) with tree branches and hanging vines as habitat
101 enrichment (Skrinyer et al. 2017). Bats had *ad libitum* access to water and food (yellow
102 mealworms *Tenebrio molitor*; Reptile Feeders, Norfolk, ON), as well as natural (hollowed tree
103 with bark) and artificial (towels) roosts. All bats selected for experiments were adult females that
104 had lived in the colony for at least 6 months (N = 18, 6 bats per treatment). During experiments,
105 bats were kept overnight in stainless steel wire mesh cages (28 x 22 x 18 cm, ¼” mesh) in an
106 indoor holding room. Bats were housed individually so they could be video-monitored five days
107 before exposure and during exposure. Behavioral and neurological changes were evaluated *in*
108 *situ* at different time points. At experimental endpoint, bats were euthanized, and their tissues
109 were extracted for biochemical analysis. All procedures were approved by the Animal Research
110 Ethics Board of McMaster University (AUP #16-06-25 and #20-05-20) and conformed to the
111 Guidelines for the Care and Use of Experimental Animals in Research published by the
112 Canadian Council on Animal Care.

113 Pesticide exposure

114 Bats (n = 6) were given an oral dose of the insecticide chlorpyrifos (CPF) mixed in oil once a
115 day at the same time (1800 hrs) for three consecutive days (72 hrs) (i.e., CPF-3d). Pesticide
116 application on large plantations might require more than three days for complete coverage, thus
117 we repeated the experiment on new bats (n = 6) using the same daily oral dose but increasing the
118 exposure to seven days (i.e., CPF-7d). In both experiments, bats were dosed by feeding them
119 mealworms (*T. molitor*), injected with a solution of CPF. In the control group (n = 6), bats were
120 fed mealworms injected only with the oil vehicle (i.e., a sham treatment). Each bat was given a

121 pesticide dose exposure of 10 $\mu\text{g/g}$ of bat body weight (BW) per day. This dose falls in the lower
122 range of concentrations that an insectivorous bat could realistically intake when foraging in
123 croplands, and corresponds to the benchmark CPF dose (BMD10: central estimate of a 10 %
124 increase in response) reported to alter 10% of plasma cholinesterase (ChE) activity (i.e., a
125 neurotoxicity biomarker) in big brown bats [12]. It is also an estimate of daily intake based on
126 the concentration of Chlorpyrifos reported from arthropods collected in crops and the
127 approximate ingestion rate normalized by body weight [28,29].

128 Behavioral tests

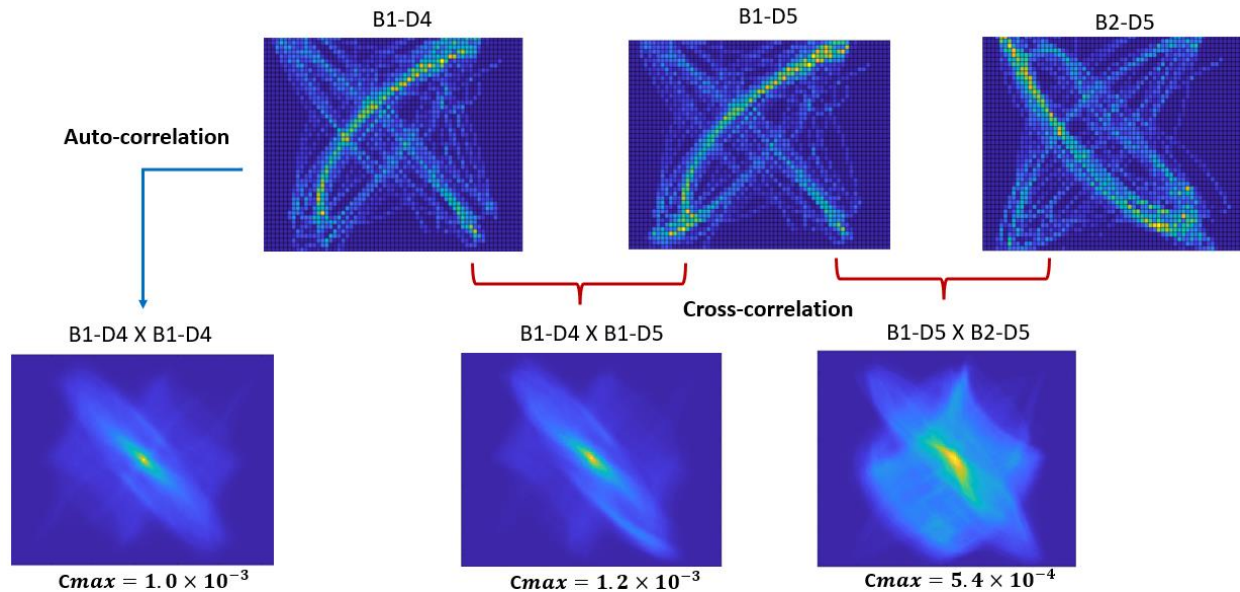
129 We evaluated three behaviors that are ecologically-relevant for free-ranging bats and logistically
130 simple to operationalize in the laboratory: (1) stereotyped flight, (2) associative memory, and (3)
131 righting reflex response. These behaviors represent different levels of cognitive complexity in
132 their execution (e.g., learning *versus* reflex response), and were intended to provide a holistic
133 understanding of the effects of CPF on different aspects of bat cognitive function. Behavioral
134 experiments were conducted at night (~2100 hrs) three hours after treatment application. The
135 sequence of testing was: (1) stereotyped flight, (2) associative memory (Y-maze), and (3)
136 righting reflex.

137 Stereotype exploration flight

138 When echolocating bats are introduced into a novel space they are reported to quickly develop
139 individual stereotyped flight patterns, such as repetitive loops along a stable trajectory [30]. We
140 evaluated bats' ability to navigate a new space by comparing the consistency of flight paths
141 between CPF-exposed and sham-treated bats. Bats were flown in a tent (Coleman Instant Screen
142 House, 11' x 11', Center Height 7'6") placed in the center of same room used for the associative
143 memory trials. We tracked flight behavior by attaching a chemiluminescent stick (2.4 mm, 0.1
144 g) to the bat's back and filming flight paths with an action camera (Go-Pro HERO10) mounted
145 inside the tent ceiling. Flight trajectories were reconstructed offline using the OpenCV library in
146 Python. We performed a quantitative analysis of the flight trajectory data in MATLAB by using
147 a two- dimensional (2-D) cross-correlation between different sets of occupancy histograms made
148 with the extracted location coordinates (Fig. 1), as described by Barchi et al. [31]. We evaluated
149 two parameters of similarity: (I) consistency of trajectory between days (cross-correlation) and
150 (II) consistency of trajectory within the same day/trial (auto-correlation). Evaluating consistency

151 between days provides information about improvement (learning) and familiarity with the space,
152 whereas evaluating the consistency of laps within a trial provides information about the
153 refinement or sharpness of the flight trajectory. In both cases, the measured parameter was the
154 maximum correlation coefficient (Cmax).

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159 Figure 1. Flight path trajectory correlation analyses reveals the consistency of flight within and
160 across trials. The three plots on the top show 2-D occupancy histograms of the reconstructed
161 trajectory for bat 1 on day 4 (B1-D4), bat 1 on day 5 (B1-D5) and bat 2 on day 5 (B2-D5).
162 Warmer colors (orange-yellow) indicate higher frequency of use. The three heat plots on the
163 bottom show the auto-correlation of the path for bat 1 on day 4 (B1-D4 X B1-D4, left), the cross-
164 correlation of paths for bat 1, days 4 and 5 (B1-D4 X B1-D5, center), and cross-correlation paths
165 for bat 1 and bat 2 on day 5 (B1-D5 X B2-D5, right). Warmer colors indicate higher correlation
166 values. The maximum correlation coefficient (Cmax) for each matrix comparison is printed
167 below each plot; with higher Cmax values indicating higher similarity between the paths.

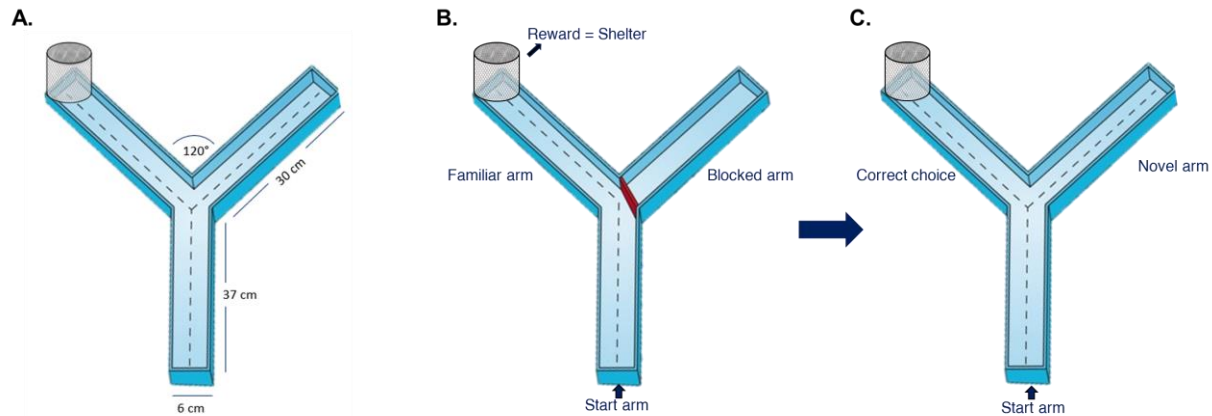
168 Associative memory Y-maze

169 To assess associative memory, we used a 2-alternative forced choice Y-maze arena. The Y-maze
170 is one of the simplest methods for assessing animal cognition and does not require rule learning,
171 extensive handling or repeated manipulation [32]. The Y-maze test has been used extensively in
172 learning and memory paradigms for rodents (Arendash et al., 2001; Conrad et al., 1997; King &
173 Arendash, 2002; Lainiola et al., 2014; Ma et al., 2007), fish (reviewed in Cleal *et al.*, 2020) and
174 bats [34–38]. Flight is, perhaps, the most common natural movement in bats, though many

175 vespertilionid bats like *E. fuscus* are adept at crawling on surfaces when locating suitable roosts
176 and exploring crevices and cavities [39].

177 Testing was conducted in the dark in a room (4.85 x 3.25 x 3.32 m) whose walls and ceiling were
178 lined with sound-attenuating foam (Sonex® Classic; Pinta Acoustic, USA). The Y-maze walls
179 and ceiling were constructed with cast-acrylic (Fig. 2A), and the maze was positioned at an
180 inclination of ~30° to encourage bat's crawling into and exploring it. An infrared camera (YI-
181 home 1080p) placed 40 cm above the maze recorded bat exploratory behavior. At the beginning
182 of each trial, a bat was placed in the start arm of the Y-maze and was permitted to freely move
183 towards the response arms. Each bat received three training trails and three test trials. During
184 training trials, one response arm of the Y-maze was blocked while the other remained open and
185 had a reward shelter at the end (Fig.2B). The shelter was a mesh cup previously familiar to the
186 bats (they are routinely weighed in this cup). Upon entering the mesh cup, the bat was permitted
187 to rest in place for 5 min before it was returned to the start arm for another training trial. During
188 the test trial, the response arm previously closed during training was opened and the bat was let
189 free to explore. Between animals, the response arm that provided with the shelter reward was
190 randomized.

191 During offline video analysis, the number of entries by a bat into each arm was noted as well as
192 the crawling speed in the start arm. Bats were scored as having entered a response arm when at
193 least two limbs were beyond the arm's border at the Y-junction. A successful entry was defined
194 as a bat selecting the response arm with the reward shelter (Fig.1C). Prior to analysis, the video
195 files were renamed, and their order was randomized so the person scoring them was blind to the
196 treatment. Due to logistical reasons the Y maze test was only conducted in bats from 2020 which
197 were exposed to CPF for seven days (CPF-7d).



198
199 Figure 2. Associative memory Y-maze behavioral testing arena. A. Schematic showing
200 dimensions of the acrylic Y-maze with a single start arm and two response arms. The dotted lines
201 illustrate exploratory paths available to the bat. B. Y-maze set up for associative memory training
202 trials with the right response arm blocked and the left response arm open and with a reward
203 shelter (mesh cup) at its end; C. Y-maze set up for associative memory testing trials with both
204 response arms open. The arm with the reward shelter is the correct choice.

205 206 Righting reflex response

207 The righting reflex test is a common behavioral assay to assess neurological function and
208 responsiveness in rodents (Franks, 2006). It has also been used as a biomarker of loss of
209 coordination in toxicological studies in bats (Clark 2016; Clark 2017). Rapidity of the righting
210 reflex correlates with the state of vestibular function, coordination, and muscular strength. To
211 examine the surface righting reflex, we placed the bat on its back on a tabletop and recorded the
212 time taken for the animal to turn over with all four limbs on the table surface. The reflex latency
213 was determined with offline video analysis (YI-home camera; 20 frames per second).

214 Cellular and molecular biomarkers

215 Tissue collection and homogenization

216 Bats were euthanized by cervical dislocation ~24 hrs after receiving the final dose of CPF for
217 their exposure regime (i.e., three or seven days). The whole brain was extracted, placed in 1.5
218 mL cryovials, immediately snap-frozen in dry ice and stored at -80 °C for further biochemical
219 analysis. For testing, samples were thawed on ice, weighed, and then transferred to a new tube
220 with ~500 µL of 0.1 M potassium phosphate buffer (pH 7.2; ~10× the volume mass). The whole
221 tissue and buffer were homogenized with a Branson Ultrasonics Sonifier™ SFX150 (intensity

222 N6) while maintaining the sample on ice. One aliquot (~250 μ L) was immediately used for ChE
223 quantification, and the rest of the homogenized tissue was reserved and stored in 1.5 ml tube at -
224 80°C for the proteomics analysis. The samples were then transported in dry ice to the Aquatic
225 Omics Lab at the Ontario Tech University, Oshawa, Ontario.

226 *Cholinesterase activity*

227 We determined ChE activity in brain tissue using a colorimetric quantification [40], adapted to
228 microplate by Guihermino (1996). Briefly, we used 1 mM acetylthiocholine and 0.1 mM 5,5'
229 dithiobis-2-dinitrobenzoic acid (DTNB) as substrate and conjugate. The reaction was measured
230 in a full spectrum spectrophotometer (BioTek Synergy HTX) at 412 nm over 15 min. Total ChE
231 activity was normalized to the amount of protein in the sample and expressed as U/mg of protein,
232 U=nmol/min. Each sample was analyzed in triplicates and the average absorbance value was
233 used. Protein quantification was conducted using the Bradford assay [41] with bovine serum
234 albumin (BSA) as the protein standard.

235 *Proteomic analysis*

236 *Protein digestion*

237 The homogenized sample was centrifuged for 15 mins at 14,000 x g at 4 °C and the supernatant
238 was transferred to a new tube and purified using an Amicon Ultra10kDa centrifugal filter. We
239 quantified protein concentration using a Bradford assay [41]. We then reduced a 50 μ L volume
240 of homogenate with the addition of 2.65 μ L of 100 mM tris(2-carboxyethyl) phosphine, as a
241 reductant, in 100 mM AB buffer, mixed using gentle vortex, and allowed to incubate at room
242 temperature for 45 min. Proteins were then alkylated with the addition of 2.8 μ L of 200 mM
243 iodoacetamide in 100 mM AB buffer, vortexed gently, and incubated in the dark at room
244 temperature for 45 min. At the end of the second incubation, 50 μ L of chemical digestion
245 solution (20% formic acid v/v) was added to each sample and vortexed for 5 s. Lid locks were
246 placed on each tube and incubated at 115°C for 30 min (VWR model 96 place heating block).
247 Samples were dried in a centrifugal evaporator (SpeedVac, Thermo-Fisher) for 40 min, stored at
248 4°C overnight, and then resuspended in 20 μ L of a solution of 95% H₂O, 5% acetonitrile and
249 0.1% formic acid. Samples were vortexed until the dried pellets were completely dissolved, and
250 then centrifuged for 10 min at 14000 g. A 20 μ L sample of the supernatant and 1 μ L of internal
251 peptide standard (H2016, Sigma-Aldrich, Oakville, ON) were added to a 2 mL screw threaded

252 HPLC vial (Chromatographic Specialties, 12 × 32 mm) containing 250 µL PP (Polypropylene)
253 bottom spring inserts (Canadian Life Sciences, 6 × 29 mm). Samples were stored at 4°C until
254 instrumental analysis.

255 A 2 µL aliquot of the peptide solution from each sample was injected and then separated by
256 reverse phase liquid chromatography using a Zorbax, 300SB-C18, 1.0 × 50 mm 3.5 µm column
257 (Agilent Technologies Canada Inc., Mississauga, ON) and Agilent 1260 Infinity Binary liquid
258 chromatographer. The Agilent 6545 Accurate-Mass Quadrupole-Time-of Flight (Q-TOF) was
259 used as the detector in tandem to the Agilent 1200 series liquid chromatography system (see
260 Appendix I for detailed instrumental methods).

261 Each analytical run included a solvent blank and a BSA digest standard (Agilent Technologies
262 Canada Inc., Mississauga, ON) injection every 10 samples to monitor baseline, carry-over, drift,
263 and sensitivity during the runtime. Samples were injected once per each bat.

264 Spectral files for each sample were analyzed using Spectrum Mill Software (Version
265 B.04.01.141). Given the limited entries available for *E. fuscus*, peptides were searched against
266 the Uniprot Reference Proteome of the bat family Vespertilionidae (ID 9431, 150,920 proteins;
267 downloaded April 2021). Proteins were manually validated and accepted when at least one
268 peptide had a score (quality of the raw match between the observed spectrum and the theoretical
269 spectrum) greater than 5 and a %SPI (percent of the spectral intensity accounted for by
270 theoretical fragments) greater than 60%; these are the manufacturer recommended settings for
271 validating results with an Agilent Q-TOF mass spectrometer. After peptides were sequenced and
272 identified by Spectrum Mill at the Tandem mass spectrometry (MS/MS) level, quantification at
273 the precursor intensity (MS1) level was performed using the data-dependent acquisition (DDA)
274 workflow in Skyline 20.2 (MacCoss Lab Software) with a score of 0.9, retention time window of
275 5 min, and 5 missed cleavages with transition settings for TOF (Pino et al., 2020).

276 [Statistical Analyses](#)

277 For the behavioural experiments, we used a GLMM (generalized linear mixed model) approach
278 to test the effects of treatment (CPF-exposed and sham-treated) and condition (before and after
279 exposure) as predictors of the different cognitive tasks. To account for the repeated measures
280 design we included the ID of the animal as a random effect. First, to evaluate the flight pattern
281 consistency within trials (auto correlation) and between days (cross correlation) among

282 treatments we ran GLMMs including time, treatment, and their interaction. We expected CPF
283 exposed bats to have no improvement in consistency after being exposed compared to sham
284 treated bats. Second, we modeled how the number of correct arm entries, total number of arm
285 entries, crawling speed (s), and reflex latency (s) was influenced by the treatment, condition, and
286 their interaction. Last, to examine the effect of exposure duration on the performance of the
287 cognitive tasks, we used GLMMs including the timepoint as fixed factor across four timepoints:
288 before exposure (BE) and one, four, and seven days after exposure (AE1, AE3, AE7), as well as
289 treatment. In all cases, we considered an effect to be statistically significant if the coefficients
290 95% confidence intervals (CI) did not include zero (0). To evaluate the differences between
291 treatments and conditions we used a Tukey pairwise post-hoc test with a Bonferroni correction.
292 These comparisons are presented as boxplots where the line represents the median and the box
293 represents the interquartile distance. The models were conducted using the package *lme4* [42] in
294 R version 3.8 [43]. The performance diagnostics of each model (linearity, collinearity, normality
295 of residuals and homogeneity of variance) were evaluated using the package *performance* in R
296 version 3.8 [43].

297 Proteomics data were sorted and manually consolidated on a spreadsheet (Microsoft Excel) and
298 statistical analyses were performed with Metaboanalyst 5.0 [44]. We replaced missing values
299 with 1/5 of the limit of detection (LOD) and normalized (using median, pareto scaling). We used
300 the normalized data and compared the treatments with a one-way ANOVA (Fisher's least
301 significant difference post-hoc test with Benjamini-Hochberg false discovery rate (FDR= 0.25)
302 and partial least squares discriminant analyses. Last, we used Revigo server to consolidate and
303 visualize the Gene Ontology (GO) enriched pathways [45]. Revigo uses multidimensional
304 scaling to reduce the dimensionality of a matrix of the GO terms pairwise semantic similarities.
305 The mass spectrometry proteomics data have been deposited to the ProteomeXchange
306 Consortium via the PRIDE partner repository with the dataset identifier PXD033247.

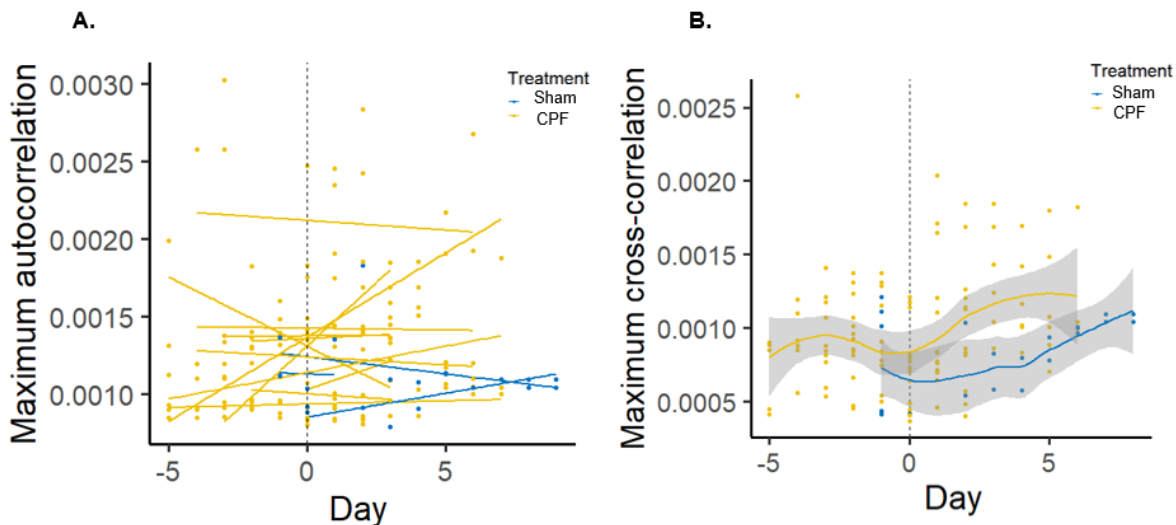
307 Finally, we used a one-way ANOVA to compare ChE activity between treatments and a Tukey
308 HSD as post-hoc test.

309 Results

310 Behavioral tests

311 Stereotype exploration flight

312 We computed cross-correlations for the flight path trajectories of CPF-exposed and sham-treated
313 bats for five days before and seven days after the treatment day (Fig. 3A). The maximum auto-
314 correlation for both sham and CPF-exposed bats increased with time suggesting an increase
315 within trial consistency in flight trajectories ($\beta = 2.31 \times 10^{-5}$; CI [$1.11 \times 10^{-6} : 4.49 \times 10^{-5}$]; Fig.
316 3A), however there was no difference among treatments ($\beta = -3.11 \times 10^{-5}$; CI [$-8.45 \times 10^{-5} : 2.14$
317 $\times 10^{-5}$]). Likewise, the maximum cross correlation between consecutive days for a given
318 individual seemed to increase with time for both treatments ($\beta = 3.41 \times 10^{-5}$; CI [$1.46 \times 10^{-5} : 5.37$
319 $\times 10^{-5}$]; Fig. 3B), however this effect was not significantly different among treatments ($\beta =$
320 5.85×10^{-6} ; CI [$-4.73 \times 10^{-5} : 5.89 \times 10^{-5}$]).
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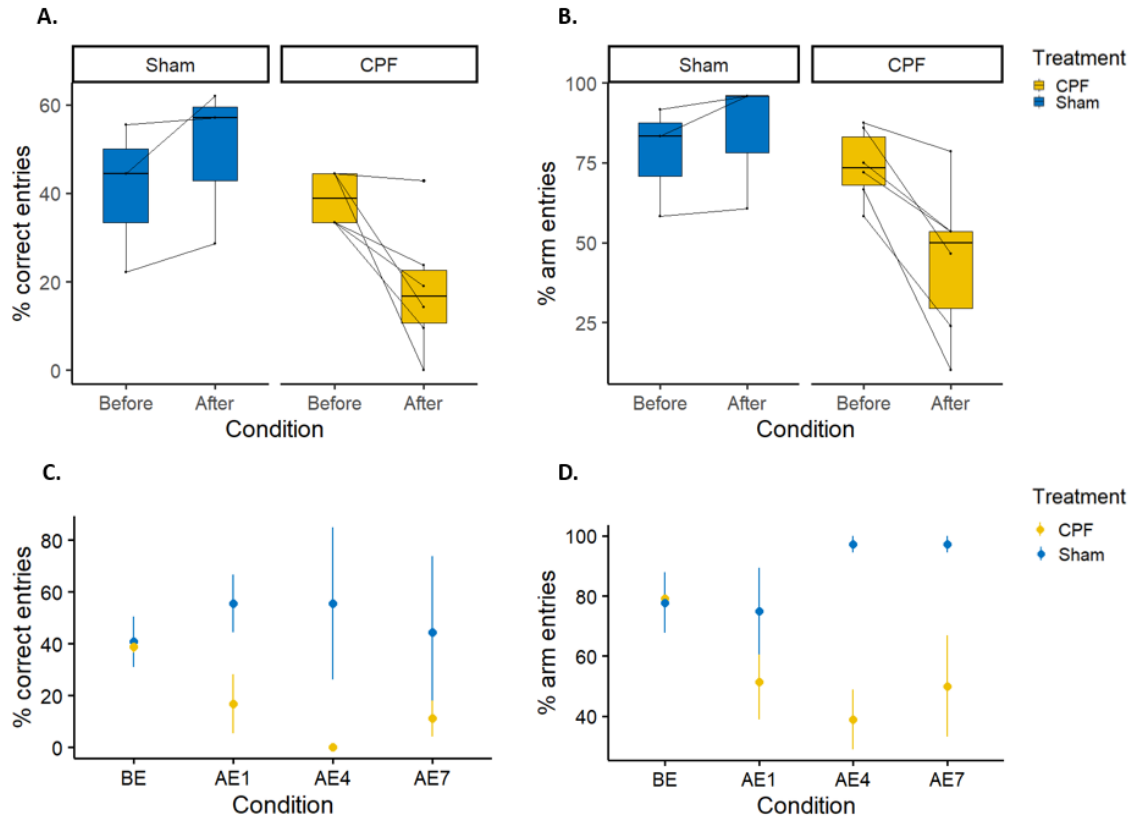
324 Figure 3. Similarity of flight trajectories in *E. fuscus* for 5 consecutive days before and after
325 treatment in CPF-exposed and sham-treated bats. A. Comparison of maximum auto-correlation
326 coefficient for flight laps within one trial; B. Comparison of maximum cross-correlation
327 coefficient among consecutive days. The dotted line at day 0 indicates when CPF or sham-
328 treatment dosing occurred; negative numbers represent the number of days before exposure and
329 positive numbers are the number of days after exposure.

330

331 Associative memory Y-maze testing

332 We found a significant difference in the proportion of correct entries in the Y maze arena
333 between the two treatment exposure conditions, before and after exposure ($\beta = 40.7$; CI [4.46 :
334 76.9]). Bats exposed to CPF showed a reduction in the number of correct entries following
335 pesticide exposure ($t_{25} = -2.7$; $p = 0.04$) whereas sham-treated bats showed no change in the
336 number of correct entries ($t_{25} = 0.73$; $p = 0.88$). When comparing different timepoints after
337 exposure to CPF (Fig. 4C), a pronounced decrease is observed three days after exposure (CPF-
338 3d) with respect to three days after exposure (CPF-7d); however, none of the interaction terms
339 were statistically significant. Similarly, the average number of arm entries, as indicator of
340 exploratory activity, differed between CPF- exposed and sham-treated bats ($\beta = -44.43$; CI [-
341 77.61 : -11.24]). Bats exposed to pesticide reduced the overall number of entries into the test
342 arms after the treatment ($t_{25} = -32.4$; $p = 0.01$) whereas sham-treated bats explored the Y maze in
343 a similar way before and after exposure to the sham treatment ($t_{25} = 0.87$; $p = 0.82$). This
344 reduction in exploration was not significantly different between 1,3 and 7 days after exposure
345 (Fig. 4D). The speed of crawling in the start arm did not differ between conditions for any of the
346 treatments ($\beta = 0.32$; CI [-25.56 : 26.21]). CPF-exposed and sham-treated bats had similar
347 median speeds (mean \pm standard error) 70.2 ± 3.3 mm/s and 74.3 ± 7.2 mm/s, respectively.

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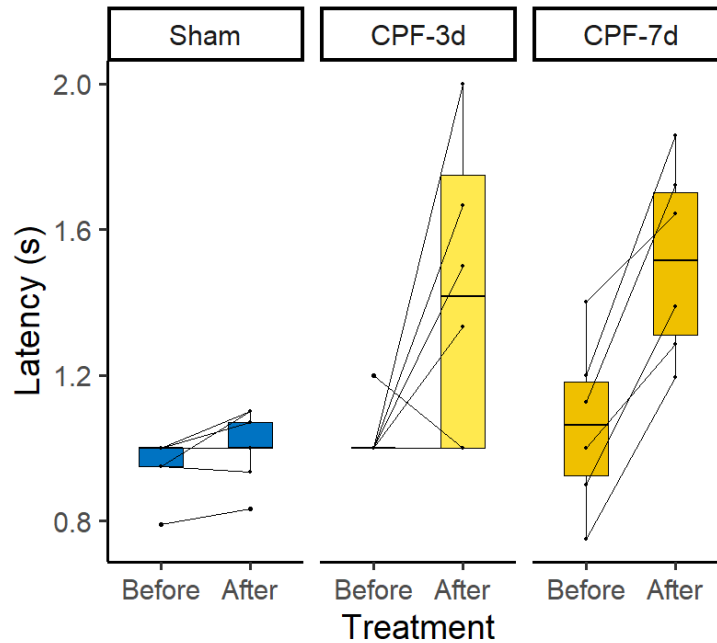


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351 Figure 4. Cognitive behavior task performance of *E. fuscus* in Y-maze testing for sham-treated
352 (blue) and bats exposed to CPF for seven days (yellow). The top row shows paired responses
353 before and after treatment, the bottom row shows the response before exposure (BE) and 1, 3 and
354 7 days after exposure (i.e., AE1, AE3 and AE7); A, C. Percent correct entries to the reward arm;
355 B, D. Percent arm entries. Thin lines in top panel connect data for the same individual.

356 Righting reflex

357 Righting reflex latency differed between the conditions, before and after pesticide exposure, for
358 CPF-exposed bats ($\beta = -0.39$; CI [-0.61 : -0.18]). Following oral treatment with CPF for three
359 days, bats took 55% longer to turn over from a supine position compared to control bats in the
360 sham-treatment ($t_{32.5} = 5.26$; $p < 0.01$; Fig. 5). Similarly, bats exposed to CPF for seven days had
361 a significantly slower reflex response relative to sham treated bats (66% longer reflex latency;
362 $t_{32.5} = 3.94$; $p < 0.01$).



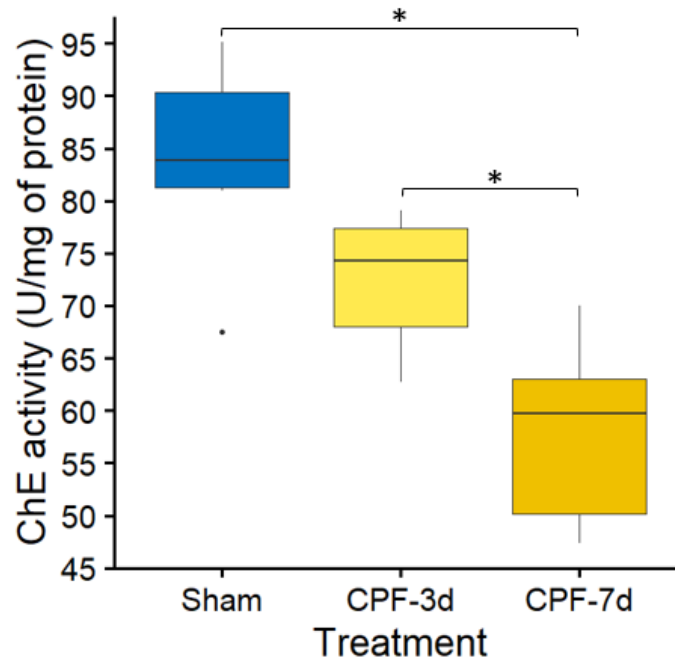
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365 Figure 5. Righting reflex response in *E. fuscus* before and after exposure to a sublethal dose of
366 chlorpyrifos (10 µL/g of BW) for three and seven consecutive days. *Thin lines* connect the reflex
367 latency data of the same individual.

368

369 Cholinesterase activity

370 Cholinesterase activity was affected by exposure to CPF ($F_{2,15} = 13.84$; $p < 0.01$; Fig. 6). Bats
371 exposed to CPF for three days showed a 15% decrease in ChE activity compared to sham-treated
372 bats (Tukey HSD: $p < 0.05$; CI = [-13.08 : -38.04]). In bats exposed for seven days, the reduction
373 in ChE activity was 30% and significantly lower than the sham-treated bats and bats exposed to
374 CPF for three days (Tukey HSD: $p < 0.05$; C.I = [-1.64 : -27.27]).

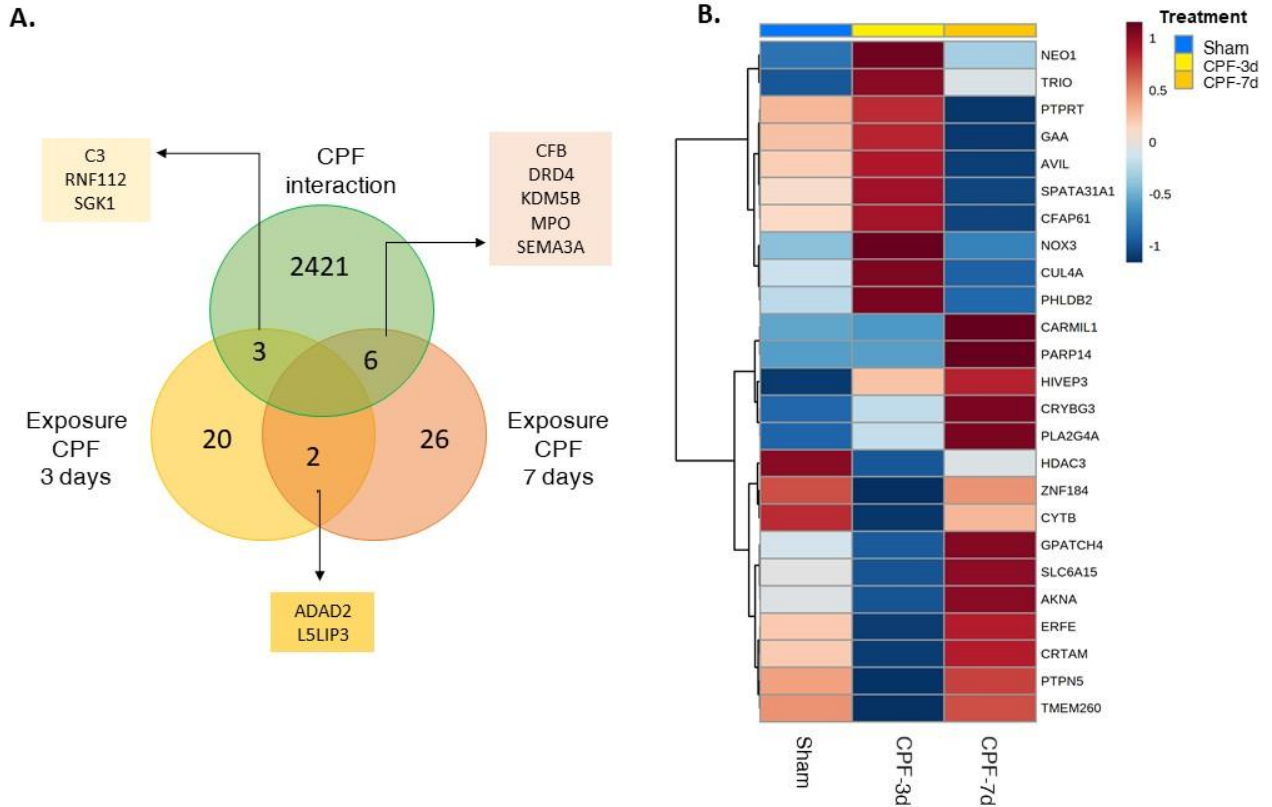


375
376 Figure 6. Brain cholinesterase (ChE) activity in *E. fuscus* exposed to a sublethal dose of
377 chlorpyrifos (10 μ L/g of BW) for three (CPF-3d) and seven (CPF-7d) consecutive days.
378

379 Proteomics

380 We identified a total of 398 proteins in bat brain samples, most of which are highly specific to
381 the nervous system in humans and mice (Human protein atlas, 2022). When comparing protein
382 profiles among the treatment groups (Sham, CPF-3d and CPF-7d), we found 53 differentially
383 abundant proteins (DAPs; $P < 0.1$); however, the statistical significance of differential expression
384 across the 53 proteins did not hold after a Benjamini-Hochberg correction ($FDR = 0.25$). Our
385 findings are nevertheless indicative of an effect with important biological implications. Overall,
386 CPF exposure altered the abundance of proteins involved in essential biological processes such
387 as: cell proliferation (e.g., HIVEP3, ZNF184), cellular metabolism (PTPRT, SGK1, GAA,
388 GPATCH4), response to stimuli (SGK1, GSTA2, CYP251) and signal transduction (CRTAM,
389 ERF2, FPR2) (Fig. 7; Supplementary Table 1).

390



391
392 Figure 7. Differential expression of proteins in brain samples of *E. fuscus* exposed to the
393 insecticide Chlorpyrifos (CPF) for three (CPF-3d) and seven (CPF-7d) consecutive days. A.
394 Venn diagram showing the number of differentially expressed proteins identified among CPF
395 exposure groups (yellow = CPF exposure for 3 days; brown = CPF exposure for 7 days and the
396 number of proteins shared with the comparative toxicogenomic database for CPF (green). B.
397 Clustering result shown as heatmap (Euclidean distance measure and Ward clustering algorithm)
398 illustrating the average expression per treatment of the top 25 differentially expressed proteins
399 (ANOVA $p > 0.1$).

Table 1. Description of relevant proteins differentially abundant in bats exposed to the insecticide Chlorpyrifos

	Protein ID	Protein name	Protein family/Type	Function	Brain expression*	FC	P-Value	References
Reduced	CUL4A	Cullin-4A	Ubiquitin-protein ligase	Regulating the cell cycle and maintenance of genomic stability Cell repair and oncogenesis	Detected in all	0.69254	0.01	[46,47]
	CYP2S1	Cytochrome P450 2S1	Oxygenase	Xenobiotic metabolism Activation of organic toxicants	Cortex	0.69254	0.05	[48,49]
	FOXP3	Forkhead box protein P3	Transcription factor	Immune tolerance by regulation of T-cells Regulates TNF- α expression Tumor growth regulation	Cerebellum	0.98708	0.91	[50,51]
	FRAS1	Fraser syndrome 1	Extracellular matrix protein	Cell communication Regulation of epidermal-basement membrane adhesion and organogenesis during development	Cortical Hippocampus Amygdala	0.71047	0.06	[52,53]
	GAA	Lysosomal alpha-glucosidase	Glucosidase	Glycogen metabolic process	Hypothalamus Cortex	0.69254	0.02	
	GSTT2	Glutathione S-Transferase Theta 2	Transferase	Antioxidant activity Detoxification Inflammation	Cerebellum Hippocampus	0.71047	0.07	[54,55]
	NOX3	NADPH oxidase 3	Oxidase	Generation of reactive oxygen species (ROS) Regulation of stress response, apoptosis, DNA damage, gene expression and differentiation	Cortex Hippocampus Inner ear Vestibular system	0.69254	0.01	[56,57]
	PTPRT	Receptor-type tyrosine-protein phosphatase T	Protein phosphatase	Regulation of synaptic formation and neuronal development	Cortex Hippocampus Olfactory bulb	0.69254	0.01	[58]
	SGK1	Serine/threonine-protein kinase	Non-receptor serine/threonine protein kinase	Cell survival and neuronal excitability	Detected in all	0.78374	0.18	[59]
	STX5	Syntaxin-5	SNARE protein	Vesicle-mediated transport Processing of β -amyloid peptide production in neuronal cells	Detected in all	0.71047	0.07	[60,61]
	VP37D	Vacuolar protein sorting-associated protein 37D	Component of the ESCRT-I complex	Regulation of membrane trafficking Vesicle fusion, vesicle recycling and neurotransmitter release	Cortex Cerebellum	0.70819	0.06	[62]
Increased	AGTRAP	Type-1 angiotensin II receptor-associated protein	Renin-angiotensin system protein	Regulation of perfusion in nervous cells Regulates permeability of Blood brain barrier	Hippocampus Amygdala	0.69254	0.05	[63]
	CARMIL1	F-actin-uncapping protein LRRC16A	Protein-binding activity modulator	Regulation of NF- κ B signaling CD28-mediated T-cell activation Regulation of migration and axon grow of neurons	Cerebellum Cortex	0.69254	0.02	[64]
	CPSF2	Cleavage and polyadenylation specificity factor subunit 2	Metallo-beta-lactamase superfamily	RNA-metabolism Regulates Alternative polyadenylation (APA) Regulates cell apoptosis and grow	Detected in all	0.69254	0.05	[65,66]
	CRYBG3	Very large A-kinase anchor protein	Beta/gamma-crystallin	Regulator of cell cycle progression Cellular apoptosis and cell proliferation in neuronal cells	Basal ganglia Olfactory bulb	0.69254	0.027556	[67]
	GPATCH4	G-Patch Domain Containing 4	RNA metabolism protein	Regulation of cellular component biogenesis e.g., ribosomes	Detected in all	0.69254	0.012687	
	HIVEP3	Transcription factor HIVEP3	Zinc finger transcription factor and adaptor protein	Transcription factor Modulation of immune response and inflammation Activation of immunoglobulin and T-cells	Cortex Hippocampus	0.69254	0.033405	[68,69]
	PLA2G4A	Cytosolic phospholipase A2	Phospholipase	Inflammation mediated by chemokines Oxidative stress response Cellular apoptosis	Detected in all	0.69254	0.04137	[70,71]
	PTPN5	Protein Tyrosine Phosphatase Nonreceptor Type 5/ STEP	Protein phosphatase	Synaptic strengthening Hippocampal long-term synaptic plasticity	Striatum Hippocampus Cortex	0.69254	0.022442	[72,73]
	RNF112	RING finger protein 112/ Neurolastin	Heterotrimeric G-protein	Neuronal differentiation, including neurogenesis and glycogenesis	Cerebellum Cortex	0.71047	0.11632	[74]
	SLC1A7	Solute Carrier Family 1 Member 7	Neutral amino acid transporter B(0)	Participates in the neurotransmitter Release Cycle Regulates Glutamate transmembrane transporter activity	Retina	0.98329	0.88204	[75]

*All the information about location of expression was taken from the Human protein atlas website. Consulted online at proteinatlas.org.

Of the DAPs, about 65% showed a uni-directional pattern of change where expression either increased or decreased as the duration of exposure increased (Fig. 8). Some proteins that increased in exposed bats participate in cellular division and apoptosis (CRYBG3, PLA2G4A), oxidative stress (PLA2G4A, CYP2S1) and inflammation (HIVEP3) processes that, if deregulated, can generate deleterious effects in nerve cells. Fewer proteins (24%) were reduced, among them, scaffold proteins like Fraser syndrome 1 (FRAS1), Serine/threonine-protein kinase (SGK1), and Receptor Protein Tyrosine Phosphatase (PTPRT). The remaining 11% of proteins showed opposite alterations in their abundance when comparing between the two exposure durations of 3 days and 7 days (Fig.7B). Among those, we detected proteins involved in DNA repair (CUL4A), cell growth rate (GPATCH4), synaptic plasticity (PTPN5) and oxidative stress (NOX3). These non-linear changes coincide with the trend observed in the associative memory evaluation where bats exposed to CPF showed the worst performance in the Y maze three days after exposure but seemed to improve after seven days of exposure (Fig.4 B).

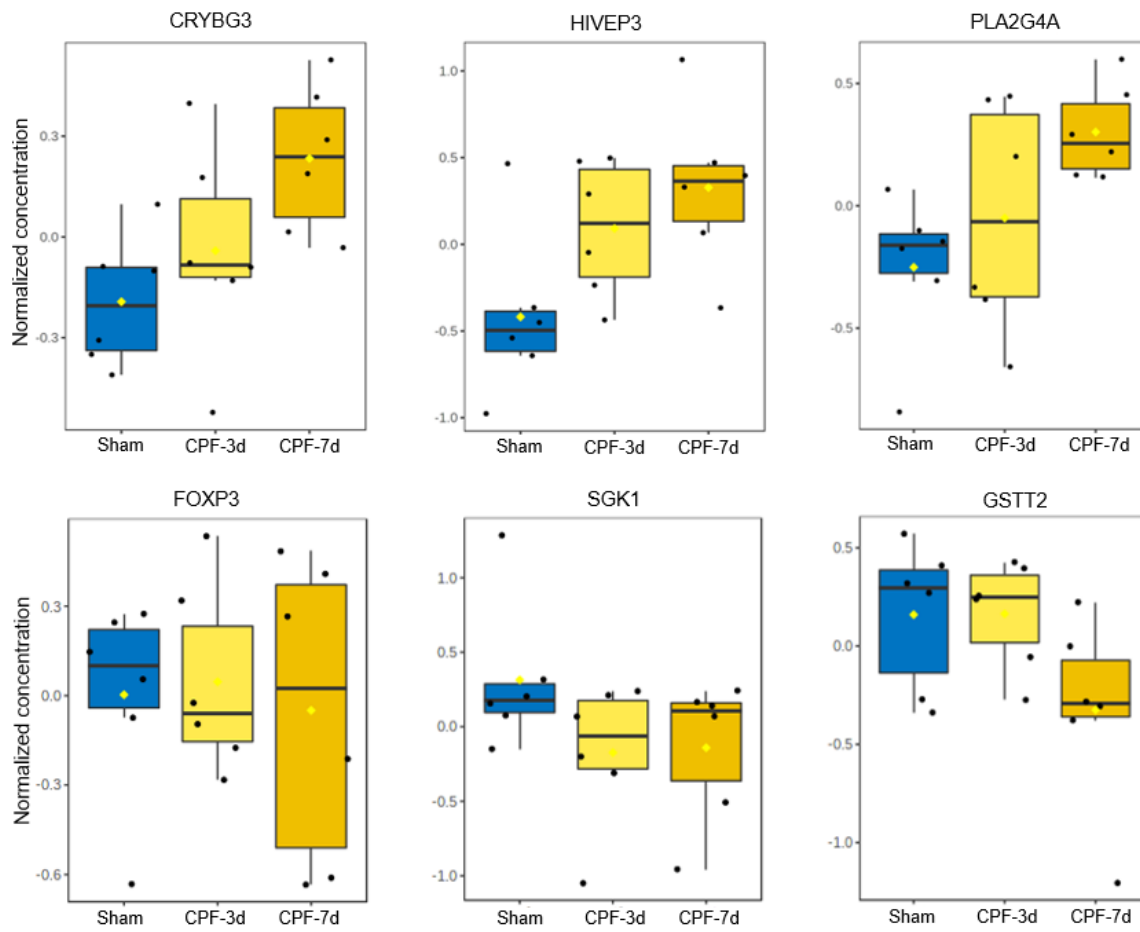


Figure 8. Six examples of differentially expressed proteins in brain samples of *E. fuscus* exposed to the insecticide Chlorpyrifos (CPF) for three (CPF-3d) and seven (CPF-7d) consecutive days. Proteins in the *top row* showed a positive linear response while proteins in the *bottom row* showed a negative linear response with the duration of the CPF exposure.

Functional enrichment analysis

Gene ontology (GO) and pathway analyses revealed that the DAPs in CPF-exposed bats resulted in the enrichment of cellular processes important for nervous system function such as synaptic transmission (e.g., PTPN5, PTPRT), oxidative stress (e.g., RNF112), and apoptosis (e.g., GRIA2, PLA2G4A) (Fig.9). Proteins associated with cellular division and death were the most abundant and significantly over-represented. Pertinent to the pesticide exposure, organophosphate metabolism was one of the significantly enriched processes, with increased abundance of related proteins in CPF-exposed bats. We also found four proteins that have been previously associated with CPF toxicity according to the comparative toxicogenomic database: glutathione S-transferase theta 2 (GSTT2), syntaxin 5 (STX5), protein tyrosine phosphatase non-receptor type 5 (PTPN5 (also known as striatal-enriched protein tyrosine phosphatase [STEP]), and excitatory amino acid transporter 5 (SLC1A7) (Fig. 8.; See Table 1 for protein descriptions).

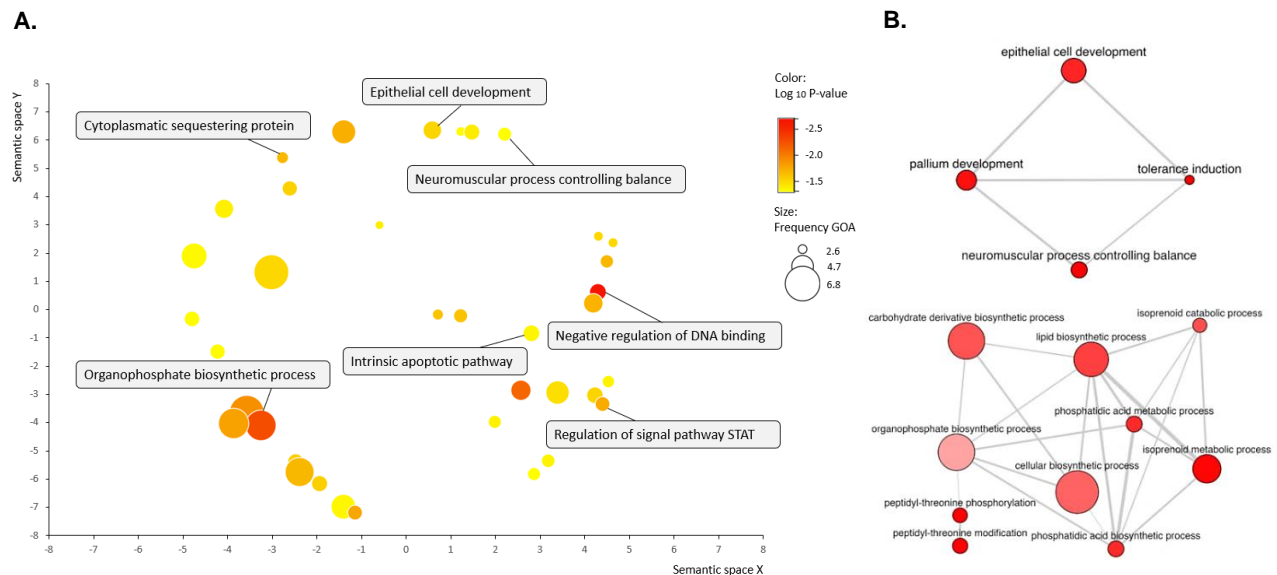


Figure 9. Enriched biological processes among the differentially expressed proteins in brain samples of *E. fuscus* exposed to the insecticide Chlorpyrifos (CPF). A. Consolidation of the enriched processes. The axes in panel A have no intrinsic meaning. B. Map network showing potential interactions among differentially enriched processes. In both panels, *bubble size*

represents the frequency of the gene ontology (GO) term in the underlying GOA database and *bubble color* indicates level of statistical significance. Highly similar GO terms are linked by edges in the graph, where the line width indicates the degree of similarity.

Discussion

Despite the critical role bats play in ecosystems coupled with decades of research into the effects of insecticides on wildlife, little is known about the risks posed to bats by insecticide use. Effects on behavior, for example, can have large implications for individual fitness and population persistence. Here we provide evidence, at the molecular and individual levels, of impairment in associative memory, which is essential for bats to effectively find food and shelter. We also found an impairment of rapidity of the righting reflex, a motor skill important for bats in maneuvers like landing. Using a proteomics approach, we detected changes in the abundance of proteins involved in neuronal function and survival, which highlights possible mechanisms underlying the observed altered behavioral phenotypes. This is the first study combining behavioral and molecular biomarkers to understand the neurotoxic effects of pesticides in a non-model mammalian species.

In several model laboratory organisms (e.g., rats, mice) there is substantial evidence that CPF neurotoxicity induces changes in behavior such as learning, fear conditioning, locomotion, and social interactions, yet few studies have reported these effects in wild/non-model vertebrates [7,12]. Most of these studies focus on ChE inhibition as the key mechanism causing disruption in behavior, and as the main proxy for OPs exposure and effects. In echolocating insectivorous bats, ChE inhibition due to OPs exposure has been reported for *Myotis lucifugus* (Clark, 1986; Clark & Rattner, 1987;) and *E. fuscus* [12], and our study found similar levels of ChE inhibition (30% for 10 $\mu\text{g/g}$) as reported previously for *E. fuscus* (40% for 10 $\mu\text{g/g}$; Eidels *et al.*, 2016) A reduction in ChE activity by more than 20% is above the reported range of natural variation in wild vertebrates, and can confidently be attributed to toxicant exposure [77]. In addition to ChE inhibition, we found enrichment of proteins involved in xenobiotic transport (TRDN), permeability of the blood brain barrier (AGTRAP), and toxicant biotransformation (CYP2S1, CYP1A2). In particular, altered expression of enzymes in the cytochrome P450 mixed-function oxidase system (CYPs) can contribute significantly to neurotoxicity by metabolizing the pesticide CPF into chlorpyrifos oxon (CPO), an active metabolite that inhibits

acetylcholinesterase (Khokhar and Tyndale, 2012; Zhou et al., 2013). An increase in the levels of these scaffold proteins provides further evidence that CPF reaches the brain and likely triggers a coping response.

Despite ChE inhibition being the most plausible and common mechanism linked to behavioral impairment, there are several other mechanisms by which CPF neurotoxicity could affect cognitive function that are often overlooked [23]. Looking at the whole brain proteome of CPF-exposed and sham-treated bats, we identified enriched cellular pathways that could underlie the non-cholinergic effects of CPF on the central nervous system. For example, we detected changes in the abundance of proteins previously associated with alterations in spatial navigation (SGK1, Ma *et al.*, 2006) and associative memory (CREB, Aggleton *et al.*, 2012; PTPN5, Zhang *et al.*, 2010). Below we discuss the roles of these and other differentially abundant proteins involved in mechanisms of neurodegeneration and neuroinflammation that could explain the altered behavioral outcomes we observed [23].

Neuroinflammation contributes to the neurotoxic effects of OPs, especially after a repeated exposure at low concentrations (reviewed in Guignet and Lein, 2019). In bats exposed to CPF, we found changes in the abundance of proteins that trigger neuroinflammation through different cellular processes: I. oxidative stress (PLA2G4A, RNF112 and NOX3); II. neuronal death (PLA2G4A, SLC1A7 and SLX5), and III. regulation of pro-inflammatory mediators (PTPRT, CARMIL1, FPR2, AGTRAP and FOXP3) (See Table 1 for details). These mechanisms can also lead to neurodegeneration and synaptic dysfunction in hippocampal regions important for spatial navigation and associative learning, like the CA1 (Cornu Ammonis 1) area and medial entorhinal cortex (MEC)[18,82]. Neural apoptosis in these hippocampal regions has been previously associated with impairment of spatial memory in bats exposed to imidacloprid, a neonicotinoid insecticide with a similar neurotoxic mode of action as CPF [5]. Additionally, we found that CPF exposure induced changes in proteins implicated in synaptic maintenance (PTPRT, SYX5) and neuroplasticity (PTPN5). Altogether, these mechanisms can explain the exposed bats' deficient performance in associative memory tasks tested in the Y-maze arena.

One protein that could be particularly relevant for bat ecology is NADPH Oxidase 3 (NOX3), an enzyme highly expressed in the mammalian inner ear and the vestibular system. This protein has been positively selected in echolocating bats, suggesting its importance in spatial navigation

using primarily auditory cues (reflected echoes) for perception [83]. Bats exposed to CPF showed upregulation of NOX3 after three days of exposure, which also corresponded to their worst performance in the associative memory test. Overexpression of NOX3, exacerbated by CPF exposure [84], can lead to hearing loss [85,86] and balance disorders due to the induction of reactive oxygen species (ROS), which causes neuronal loss in the central auditory system.

We have discussed some of the main protein changes that we observed and that are likely related to mechanisms of CPF neurotoxicity and impairment of behavior in bats. It is important to highlight that for some of the proteins the direction of the effects was not consistent across the two exposure durations. For some proteins, the direction of change after seven days of CPF exposure was opposite to the effect observed after three days of exposure (Fig. 8). In a multi-dose study, second and subsequent doses were found to change the biomarker responses because the system might not return to equilibrium between doses [87]. Thus, responses observed at later stages can reflect mechanisms of recovery or cellular repair rather than neurotoxic effects. On the other hand, homeostatic feedback mechanisms can prevent a sustained, unidirectional response and would tend to return proteins to baseline levels, especially when persistently altered expression can have severe consequences for cell integrity and survival. Given that we observed different protein patterns at three and seven days of exposure, and that the effects in some molecular pathways appear and disappear relatively quickly, we suggest that protein expression needs to be examined more frequently and with higher resolution to eliminate the possibility that no effect was observed when in fact changes may have occurred as an early response to exposure. In particular, late measurement when severe cellular damage has occurred may provide little mechanistic information on the toxicant mode of action [88].

While a standardized Y-maze assessment arena is an informative short-term test of learning and spatial memory, a longer-term and perhaps more ecologically relevant test for spatial navigation in bats is the ability to adopt stereotyped flight patterns when exploring novel space [31].

Studying stereotyped flight behavior provides insight into the interaction between echolocation, spatial memory, and flight control, and could help to elucidate how pesticides affect complex behavior. Stereotyped flight was found to be affected by the neonicotinoid imidacloprid, [5].

Contrary to our expectations based on previous findings, we found no difference in the consistency of or improvement in the flight trajectory over time in bats exposed to CPF (Fig. 1). Given the robust evidence of neurotoxicity that we found at the molecular level (Fig. 7, 8, and 9),

and its correlation with other behavioral impairment (Fig. 2), we cannot conclude that CPF does not affect bat ability to sustain stereotyped flight. One important difference in the Hsiao et al. (2016) study is that they used newly caught bats; the relatively lower stamina of our captivity-acclimatized experimental animals could have introduced noise in the evaluation of their flight performance. Captive bats may be less motivated to initiate flight, and, in some cases, bats were unable to sustain continuous flight likely due to their increased weight in captivity, which might have influenced the accuracy of our flight trajectory estimates.

The righting reflex response is another important behavioral endpoint widely used in toxicology to assess overt neurotoxic effects of OPs pesticides. For example, rodents exposed to CPF decrease their righting performance or lose the reflex altogether [89–92]. Previous studies in bats have also found the righting reflex response to be a sensitive indicator of OPs neurotoxicity, with righting latency being positively correlated with pesticide dose [12,76,93]. Consistent with these findings, we observed a significant delay in the righting reflex response of *E. fuscus* exposed to CPF, with treated bats on average taking 50% longer to execute the maneuver compared to control group bats (Fig 5). Impairment of this reflex can have detrimental implications for survival because righting maneuvers are essential for landing—requiring bats to reorient their body and half-summersault upside down — and correcting flight trajectory after catching prey [94]. Molecular mechanisms involved in synaptotoxicity could contribute to deficient cell signaling to elicit or properly coordinate this reflex response. It is important to note that while our results are consistent with previous studies in bats, the magnitude of the effect was smaller and, unlike previous studies, we did not observe other motor impairments such as the inability to fly or the presence of tremors and seizures [12,76,93]. The absence of these responses is most likely explained by the lower concentration we used to expose bats compared to previous studies that used 2-60 times greater doses.

Conclusions

Pesticide use is a potential threat for bat populations that has been overlooked and can represent a major conservation problem in agriculture-dominant regions. Although the main mode of action of common pesticides like OPs has been widely studied, other physiological mechanisms for off-target neurotoxicity, and their consequences for individual and population health in

wildlife, are still poorly understood. We found that CPF is a potent neurotoxicant that leads to behavioral impairments in bats, at low concentrations of exposure, likely due to neuronal dysfunction caused mainly by oxidative stress and neurochemical disruption. Integrating molecular and whole-animal individual responses enabled us to link neurotoxic effects with neurobehavioral outcomes. The behavioural effects we observed may compromise bat survival and lead to population declines with concomitant losses of ecological functionality and ecosystem services [3,76,95]. Our findings highlight the importance of considering endpoints other than mortality, or single molecular biomarkers when assessing the potential impact of pesticides on wildlife.

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