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1 # *C. elegans* pharyngeal pumping provides a whole organism bio-assay to
2 investigate anti-cholinesterase intoxication and antidotes.

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23 ## Research Highlights

24 - *C. elegans* pharyngeal pumping inhibition by organophosphates
25 correlates with worm acetylcholinesterase inhibition by the anti-
26 cholinesterases.
27 - The recovery of the pharyngeal function in *C. elegans* in the presence of
28 obidoxime is due to the recovery of the acetylcholinesterase function
29 after anti-cholinesterase intoxication.
30 - The pharyngeal neuromuscular function represents a quantitative bio-
31 assay for investigation of anti-cholinesterase toxicity and recovery with
32 excellent 3Rs potential.

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34 **Abstract**

35 Inhibition of acetylcholinesterase by either organophosphates or carbamates causes anti-
36 cholinesterase poisoning. This arises through a wide range of neurotoxic effects triggered by the
37 overstimulation of the cholinergic receptors at synapses and neuromuscular junctions. Without
38 intervention, this poisoning can lead to profound toxic effects, including death, and the incomplete
39 efficacy of the current treatments, particularly for oxime-insensitive agents, provokes the need to find
40 better antidotes. Here we show how the non-parasitic nematode *Caenorhabditis elegans* offers an
41 excellent tool for investigating the acetylcholinesterase intoxication. The *C. elegans* neuromuscular
42 junctions show a high degree of molecular and functional conservation with the cholinergic
43 transmission that operates in the autonomic, central and neuromuscular synapses in mammals. In fact,
44 the anti-cholinesterase intoxication of the worm's body wall neuromuscular junction has been
45 unprecedented in understanding molecular determinants of cholinergic function in nematodes and
46 other organisms. We extend the use of the model organism's feeding behaviour as a tool to investigate
47 carbamate and organophosphate mode of action. We show that inhibition of the cholinergic-
48 dependent rhythmic pumping of the pharyngeal muscle correlates with the inhibition of the
49 acetylcholinesterase activity caused by aldicarb, paraoxons and DFP exposure. Further, this bio-assay
50 allows one to address oxime dependent reversal of cholinesterase inhibition in the context of whole
51 organism recovery. Interestingly, the recovery of the pharyngeal function after such anti-
52 cholinesterase poisoning represents a sensitive and easily quantifiable phenotype that is indicative of
53 the spontaneous recovery or irreversible modification of the worm acetylcholinesterase after
54 inhibition. These observations highlight the pharynx of *C. elegans* as a new tractable approach to
55 explore anti-cholinesterase intoxication and recovery with the potential to resolve critical genetic
56 determinants of these neurotoxins' mode of action.

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65 **1. Introduction**

66 Organophosphates and carbamates are potent acetylcholinesterase inhibitors (Colovic, Krstic et al.
67 2013, Tattersall 2018). This enzyme is key in terminating the cholinergic transmission that controls
68 neuromuscular junction and important central synapse function (Koelle 1954, Massoulie, Pezzementi
69 et al. 1993). This mode of action has led to the development of these compounds for widespread use
70 as pesticides based on the central role of cholinergic transmission in the animal and plant parasitic life
71 cycle (Takahashi and Hashizume 2014). This widespread use of anti-cholinesterases as pesticides has
72 an associated human intoxication issue. At least two million cases of poisoning per year result in an
73 estimated 200,000 deaths (Jeyaratnam 1990, Eddleston and Phillips 2004, Gunnell, Eddleston et al.
74 2007, Eddleston and Chowdhury 2016). Additionally acetylcholinesterase inhibitors with high human
75 toxicity were developed as nerve agents for chemical warfare and terrorism (Colovic, Krstic et al. 2013,
76 Worek, Wille et al. 2016).

77 The toxicological effect of organophosphates and carbamates is exerted through the covalent
78 modification of acetylcholinesterase (AChE) (Colovic, Krstic et al. 2013, Tattersall 2018). The anti-
79 cholinesterase drugs are orientated in the catalytic centre of the enzyme in a similar manner to
80 acetylcholine (Dvir, Silman et al. 2010). When the molecule is positioned at the catalytic triad (Ser-
81 Glu-His), the phosphorylation (OP) or carbamylation (carbamate) of the serine leads to inactivation of
82 the AChE (Dvir, Silman et al. 2010). This inhibition results in the accumulation of the acetylcholine in
83 the synaptic cleft causing the potential continued agonist activation of the two distinct classes of
84 cholinergic receptors, muscarinic and nicotinic (Albuquerque, Deshpande et al. 1985). This
85 overstimulation of the cholinergic target cells causes a wide range of neurotoxic effects. The first
86 manifestations of the associated cholinergic syndrome cause autonomic disturbances including
87 excessive sweating, lacrimation, salivation as well as cramps, bradycardia and miosis (Jokanovic and
88 Kosanovic 2010, Tattersall 2018). Fatality occurs primarily due to disruption of the respiratory centres
89 in the brain and/or transmission failure at the respiratory muscles (Jokanovic and Kosanovic 2010).

90 After enzyme inactivation, spontaneous reactivation occurs via hydrolysis of the bond created
91 between the enzyme and the inhibitor molecule and enables the re-use of the AChE (Colovic, Krstic et
92 al. 2013). This reversibility is important in managing recovery from intoxication. The rate at which it
93 happens depends on the organophosphate or carbamate molecule and shows strong variation in the
94 rate between distinct classes of anti-cholinesterase (Worek, Thiermann et al. 2004). However, the
95 chemistry of the organophosphate attack is complicated by an ancillary reaction termed aging that
96 leads to an irreversible inhibition in the OP-inhibited AChE (Wiener and Hoffman 2004, Colovic, Krstic
97 et al. 2013). The dealkylation of any side chain of the conjugated OP creates a bond resistant to

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98 hydrolysis between the inhibitor and the catalytic serine (Li, Schopfer et al. 2007). It is a time-
99 dependent reaction whose rate is extremely variable depending on the chemical structure of the
100 intoxicating OP molecule (Worek, Thiermann et al. 2004).

101 Artificial ventilation is used to preserve breathing while the diaphragm neuromuscular junctions
102 undergo a more long-term recovery involving hydrolysis and resynthesis of the otherwise dead
103 molecule. This mitigation is supported by treatment with oximes, which are potent nucleophile
104 molecules able to hydrolyse and reverse the AChE inhibition. In addition, the antidotes to intoxication
105 are supported by low dose atropine and benzodiazepine treatment (Eddleston and Chowdhury 2016).
106 Although this treatment has been used for the last 60 decades, it is deficient in several aspects
107 (Buckley, Karalliedde et al. 2004). Firstly, atropine is a nonspecific competitive antagonist of
108 muscarinic receptors, meaning that overstimulation of the target cell can still occur throughout the
109 nicotinic receptors. Furthermore, there has not been a dose-response study to identify the optimal
110 dose of atropine and the excessive administration might result in anti-muscarinic toxicity with fatal
111 consequences (Eddleston, Buckley et al. 2004, Eddleston and Chowdhury 2016). Secondly, the success
112 of reactivating AChE by oximes depends on which of the various OP molecules has produced the
113 inhibition. For example, obidoxime seems to be more efficient for reactivating AChE after the
114 inhibition of OP pesticides but not nerve agents. The efficiency of 2-pralidoxime is demonstrated after
115 the inhibition of AChE with sarin or VX but not by soman or tabun. Lastly, there is not any reactivator
116 able to recover the AChE activity after the aging reaction (Worek, Thiermann et al. 2004).

117 The limitations of the current treatment, poor health condition of the surviving victims and the
118 fatalities reported have become a major public health concern (Jeyaratnam 1990, Konradsen 2007).
119 In this scenario, identifying model organisms, which replicate the biological manifestation of OP
120 toxicity, is key to develop alternative strategies. Mammal animal models have been used to address
121 this situation, with species ranging from small rodents to large mammals, including non-human
122 primates (Pereira, Aracava et al. 2014). The signs and LD50 values of anti-cholinesterase poisoning in
123 these models are well correlated to the IC50 of AChE inhibition in both brain and blood samples (Sivam,
124 Hoskins et al. 1984, Fawcett, Aracava et al. 2009). However, since the development of the current
125 treatment, between 1950s and 1960s, it has not been significantly improved. Taking into consideration
126 this fact as well as the 3Rs principles for animal research (Prescott and Lidster 2017, Balls and Combes
127 2019), the genetically tractable model organism *C. elegans* is proposed in this study. It has been widely
128 used in neurotoxicological studies including organophosphates (Cole, Anderson et al. 2004, Melstrom
129 and Williams 2007, Rajini, Melstrom et al. 2008, Jadhav and Rajini 2009, Lewis, Szilagyi et al. 2009,
130 Vinuela, Snoek et al. 2010, McVey, Mink et al. 2012, Leelaja and Rajini 2013, Lewis, Gehman et al.
131 2013). This is advantaged by highly conserved molecular pathways between the nematode and

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132 humans. There is a rich cholinergic signalling network in which acetylcholine controls the worm's
133 nervous system and is essential for neuromuscular transmission (Rand 2007, Pereira, Kratsios et al.
134 2015). The cholinergic neuromuscular transmission, which excites distinct muscles, underpins
135 biologically critical functions such as locomotion, egg-laying and the feeding behaviour (Rand 2007,
136 McVey, Mink et al. 2012). As in mammals, acetylcholinesterase is key in terminating the cholinergic
137 signal to prevent hyperstimulation. The three *C. elegans* acetylcholinesterases are orthologous to the
138 three human acetylcholinesterase isoforms (Arpagaus, Combes et al. 1998, Combes, Fedon et al. 2000,
139 Selkirk, Lazari et al. 2005). In particular, the catalytic centre of the nematode enzyme is highly
140 conserved to mammals and harbours the key amino acids involved in the inhibition and aging
141 reactions (Combes, Fedon et al. 2000).

142 *C. elegans* exposed to anti-cholinesterases exhibits easily scored defects in behaviours (McVey, Mink
143 et al. 2012). These include hypercontraction of body wall muscles that results in the paralysis of the
144 worms (Cole, Anderson et al. 2004, Rajini, Melstrom et al. 2008, McVey, Mink et al. 2012). In fact,
145 toxicity ranks for investigated anti-cholinesterases are comparable in their inhibition to relative
146 potencies identified in mammalian models, consistent with the highly conserved catalytic site (Cole,
147 Anderson et al. 2004, Rajini, Melstrom et al. 2008).

148 We have investigated how anti-cholinesterases act on the high rate of pharyngeal pumping that
149 worms use to feed on bacteria (Avery 1993, Avery and Shtonda 2003, Niacaris and Avery 2003). Here,
150 we show that whole organism measurement of pharyngeal movements represents a sensitive
151 phenotype that allows us to use it as a bio-assay for the whole organism effects of OP intoxication.
152 Furthermore, the inhibition of nematode acetylcholinesterases was better correlated to the inhibition
153 of the pharyngeal pumping than to the paralysis of the body wall muscles. We validated the
154 pharyngeal pumping as a tool to probe spontaneous recovery as well as the reversible and irreversible
155 inhibition associated with aging. This was confirmed by biochemical analysis of the nematode
156 acetylcholinesterase activity. Thus, the pharynx offers a powerful bio-assay to investigate mode of
157 action and approaches by which chemical mitigation can be used to treat intoxication. The possibility
158 to resolve genetic determinants that might act beyond the primary mode of action of the
159 organophosphate in *C. elegans* suggests the organization of pharyngeal pumping might provide a
160 route to allow novel understanding of these important neurotoxins.

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162 ## 2. Materials and Methods

163 ### 2.1. *C. elegans* maintenance

164 All the experiments were performed using N2 Wild-type *C. elegans* strain obtained from
165 Caenorhabditis Genetics Center (https://cgc.umn.edu/) and maintained under standard conditions
166 (Brenner 1974). Briefly, nematodes were growth at 20ºC on Nematode Growth Medium (NGM) agar
167 plates seeded with *E. coli* OP50 as source of food.

168 ### 2.2. Drug stocks

169 Carbamate (aldicarb) and organophosphates (paraoxon-ethyl, paraoxon-methyl and DFP) were
170 acquired from Merck and dissolved in 70% ethanol and 100% DMSO, respectively. The oximes,
171 obidoxime and 2-pralidoxime, were provided by DSTL Porton Down (UK) and dissolved in distilled
172 autoclaved water. The drug stocks were kept at 4ºC, as manufacturer recommended temperature, in
173 a locked cabinet according with standard security protocols. Dissolved compounds were used within
174 one month or discarded.

175 Acetylthiocholine iodide (ATCh) and 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) were obtained from
176 Merck (https://www.sigmaaldrich.com/united-kingdom.html) and dissolved in phosphate buffer 0.1
177 M pH7.4 directly before use.

178 ### 2.3. Behavioural assays

179 All behavioural experiments were performed on a standard developmental stage: young
180 hermaphrodite adults (L4 + 1 day) at room temperature (20ºC). Worms were allowed to develop from
181 eggs at 20ºC through the larval stages L1, L2, L3. Worms were viewed under a Nikon SMZ800 binocular
182 zoom microscope and were recognized as L4 by the temporary appearance (8 hours window) of the
183 vulva saddle. These worms were selected the day before the experiment and placed on fresh OP50
184 seeded plates. They were used 16-24 hours after as L4 +1.

185 Anti-cholinesterase containing plates were prepared the day before of each experiment by adding an
186 aliquot of a concentrated stock in the melted NGM, tempered after heating to approximately 60ºC. 50
187 µl of OP50 *E. coli* bacteria one $OD_{600\ nm}$ was dropped on the plate when the media was solidified. After
188 1 hour in the fume hood, the dried bacterial plates were sealed and kept in dark at 4ºC until next day.
189 Plates were left at room temperature for at least 30 min before starting the experiment. There was
190 no observable change in the bacterial lawn of anti-cholinesterase-containing and control plates,
191 therefore no effect of the anti-cholinesterase on the *E. coli* growth was discernible (Kudelska, Lewis et
192 al. 2018). The final concentration of vehicle in the behavioural assay was 0.07% ethanol for aldicarb-
193 containing plates and 0.1% of DMSO for organophosphate-containing plates. Control plates contained

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194 the same concentration of vehicle than assay plates. Neither vehicle concentrations alone had any
195 effect in the phenotypes tested.

196 Nematodes were picked onto the bacterial lawn 10 minutes before starting observations. Worms that
197 left the patch of food during the experiment were picked back to the bacterial lawn. They had to be
198 on the lawn for at least 10 minutes before the start of observations to be counted (Dalliere, Bhatla et
199 al. 2016).

200 **2.4. Intoxication with aldicarb**

201 Experiments were performed in 6-well plates containing a final NGM volume of 3 ml. Aldicarb was
202 added to the assay at final concentration between 2 µM and 500 µM. Vehicle control plates were used
203 as control. Young adult worms were placed on aldicarb and non-aldicarb containing plates where the
204 pharyngeal pumping, percentage of paralysed worms and body length was scored at indicated times.

205 Paralysis and body length were scored as previously described (Mahoney, Luo et al. 2006, Mulcahy,
206 Holden-Dye et al. 2013). Briefly, nematodes were picked onto the bacterial lawn containing either
207 aldicarb or vehicle control. Paralysis was scored by quantifying the number of animals not moving out
208 the total of worms on the plate at indicated times. These snapshots involved scoring for 30 secs.
209 Nematodes were considered paralysed when no movement was detected after prodding three times
210 with a platinum wire (Mahoney, Luo et al. 2006). To measure body length, images of the worms were
211 taken at the specified times. These images of the nematodes were binarized and skeletonized using
212 ImageJ software. The length of the skeleton was used to determine the body length of the nematodes
213 (Mulcahy, Holden-Dye et al. 2013).

214 Pharyngeal pumping on food in the presence of aldicarb was scored at indicated times after
215 transferring worms to aldicarb or vehicle control plates (10 min, 1 h, 2 h, 6 h, and 24 h after picking
216 onto assay plates). Pumping was quantified by counting the number of grinder movements observed
217 under binocular microscope. The pump rate was quantified for a minimum of 3 minutes per worm at
218 each time point and the mean was used as pumps per minute.

219 The estimates of IC50 were made by measuring the body wall and pharyngeal function at varying drug
220 concentrations after 24 hours incubation relative to worms placed on drug free vehicle control plates.
221 The percentages of inhibition relative to these controls were used to estimate IC50.

222 **2.5. Intoxication with the organophosphates paraoxon-ethyl, paraoxon-methyl and DFP**

223 For pharyngeal intoxication assays with organophosphates, the same procedures were utilized as
224 indicated in section 2.4., with the exception of DFP. This organophosphate equilibrates across the
225 individual wells of the 6-well culture plates. This was evidenced by the inhibition of pumping and

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226 paralysis of worms placed on non-DFP wells adjacent to DFP laced agar (data not shown). This potent
227 cross-contamination by DFP concentrations precluded the use of 6-well plates. Therefore, 9 cm Petri
228 dishes were used containing a final volume of 20 ml NGM. DFP was added to the melted NGM as
229 mentioned above to obtain the indicated final concentrations between 2 µM and 500 µM. Non-DFP
230 containing plates were used as control. After solidification, 200 µl of *E. coli* OP50 $OD_{600\ nm}$ = 1 was
231 spread evenly over the complete surface of the NGM. This full food coverage was needed to mitigate
232 the potent drive for worms to leave food that was particularly strong in the case of the DFP treatments
233 (data not shown). Seeded plates were incubated for 1 h and then they were kept until the next day as
234 mentioned in section 2.3.

235 **2.6. Recovery from organophosphate intoxication**

236 To study the recovery of pharyngeal pumping after organophosphate intoxication, L4 + 1 worms were
237 intoxicated on organophosphate-containing plates for 24 hours. The intoxicating concentration was
238 calculated based on estimating the lowest concentration that gave the maximal inhibition of pumping
239 after 24 hours of exposure. After incubation on organophosphate laced plates for 24 h, the nematodes
240 were transferred onto either non-drug containing plates or oxime-containing plates. From here, the
241 recovery from full inhibition was measured by recording the pump rate at indicated times after being
242 placed on no-drug or oxime plates. Oxime plates were poured, seeded with OP50 and stored using the
243 protocol mentioned in section 2.3. Neither obidoxime nor pralidoxime alone had an effect on the
244 pharyngeal pumping rate at concentrations between 0.5 mM and 2 mM (Supplementary figure 1).

245 **2.7. Biochemical assays**

246 Total worm homogenates were generated from synchronized L4/adult worms. For this, 12 gravid
247 worms were maintained for 4 h on OP50 seeded 5.5 cm plates, in which time they accumulated freshly
248 laid eggs. The adult worms were removed and plates were incubated 3 days at room temperature.
249 This generated approximately 250 age-synchronized L4/adults on bacteria depleted plates. Worms
250 from a minimum of 40 plates (approx. 10,000 worms) were harvested and washed three times with
251 0.1 M phosphate buffer pH7.4 in order to remove all the remaining bacteria. Nematodes were
252 transferred to a glass homogenizer and incubated for 30 min on ice with a final concentration of 0.15%
253 of n-octyl-glucoside as detergent to permeabilize the cuticle and release cellular content (Blaxter
254 1993). The n-octyl-glucoside did not alter the acetylcholinesterase activity (data not shown). Mouse
255 brain homogenate was used in parallel to validate the acetylcholinesterase activity quantification
256 protocols in *C. elegans* and compare with previously published data. To generate mouse forebrain
257 homogenate, freshly dissected tissue was homogenized in 10 volumes of phosphate buffer (w/v). This

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258 was kindly provided by Aleksandra Pitera (Southampton University, UK). Worm/mouse protein
259 homogenate was stored at -80ºC until use when they were defrosted on ice.

260 Acetylcholinesterase activity was measured using a modified colorimetric Ellman's assay (Ellman,
261 Courtney et al. 1961). The assay mixture contained 0.2 mg/ml of worm/mouse homogenate
262 comprising the AChE enzyme, 0.48 mM acetylthiocholine (ATCh) as substrate and 0.32 mM 5,5-dithio-
263 bis-(2-nitrobenzoic acid) (DTNB) as chromophore in a final volume of 200 µl of 0.1 M phosphate buffer
264 pH7.4. The increase in absorbance at 410 nm was measured at 1 min intervals for 15 min at room
265 temperature using a FLUOstar Optima microplate reader (BMG Labtech). The change in absorbance
266 against time due to the production of 5-thio-2-nitro-benzoic acid and its extinction coefficient was
267 utilized to calculate the acetylcholinesterase activity (µmoles/min). This was normalized to the protein
268 content of worm/mouse homogenate determined by standard Bradford protocol (Bradford 1976). The
269 enzyme activity in the homogenate was expressed as µmoles/min/mg protein.

270 **2.8. Acetylcholinesterase activity after whole worm aldicarb intoxication**

271 To estimate the acetylcholinesterase activity after aldicarb intoxication, nematodes were
272 synchronized as in section 2.7. When worms reached the L4/adult stage, an aliquot of 12 µl aldicarb
273 stock was added to the worm-containing plates (12 ml) to generate the indicated final aldicarb
274 concentration of 50 µM or 500 µM. Control plates were made by adding 12 µl of 70% ethanol. A
275 minimum of 40 plates were used per condition. After 24 hours of incubation at room temperature the
276 control and aldicarb intoxicated worms were harvested, washed and treated to generate the worm
277 homogenate. Nematodes were kept on ice during the whole process to prevent recovery of the
278 acetylcholinesterase activity through reversibility of the reaction. Acetylcholinesterase activity assays
279 were carried out directly after the worm protein extraction.

280 **2.9. Acetylcholinesterase activity of worm/mouse brain homogenate after inhibition by**
281 **organophosphates**

282 A stock solution of organophosphate was appropriately diluted in 0.1 M phosphate buffer pH7.4 just
283 before the experiment to ensure a low concentration of DMSO in the final dilution for
284 acetylcholinesterase activity quantification. The final concentration of vehicle in the biochemical assay
285 was 0.000025%.

286 Worm/mouse brain homogenate in phosphate buffer (0.1 M pH7.4) as described above was placed in
287 a 96-well plate. At 0 min, 15 min, 30 min, 35 min, 40 min, 43 min, 44 min, 44.3 min, 44.6 min and 45
288 min, these volumes were supplemented with organophosphate to the indicated final concentration in
289 50 µl final volume. This incubation contained 120 µg of worm/mouse protein and 1 µM
290 organophosphate. After 45 min, acetylcholinesterase activity assay was scored for all the samples as

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291 described above (section 2.7) by addition of DNTB and acetylthiocholine in a final volume of 200 μl
292 with 0.1 M phosphate buffer pH7.4. This gave a time series in which the time of incubation with the
293 organophosphate was 20 s, 40 s, 1 min, 2 min, 5 min, 10 min, 15 min, 30 min and 45 min. An
294 acetylcholinesterase assay without the presence of the OP was used as control.

295 Either single or double decay exponential curve was fitted to these time courses to determine the
296 best-fit mode of inhibition of acetylcholinesterase by each organophosphate.

297 **2.10. Acetylcholinesterase reactivation after inhibition with organophosphate drugs**

298 Organophosphate-inhibited acetylcholinesterase was prepared by incubating 2.4 mg/ml of
299 worm/mouse homogenate with 2 μM of any organophosphate in a final volume of 320 μl for 1 hour
300 at room temperature. The mixture was centrifuged at 14000 rpm for 30 min and the supernatant was
301 discarded to remove the excess of organophosphate. The pellet containing the inhibited
302 acetylcholinesterase was resuspended with 320 μl of phosphate buffer (0.1 M pH7.4) and maintained
303 at room temperature. For control, non-exposed 2.4 mg/ml worm/mouse protein was used in parallel.
304 Following organophosphate removal, aliquots were taken at subsequent time intervals to determine
305 acetylcholinesterase activity after incubating the mixture 1 min in the presence or absence of 100 μM
306 obidoxime (Worek, Thiermann et al. 2004). The concentration of obidoxime used was previously
307 described to research spontaneous recovery and aging reaction in human acetylcholinesterase
308 inhibited by paraoxon-ethyl, paraoxon-methyl or DFP (Worek, Thiermann et al. 2004).

309 Acetylcholinesterase activity that does not recover upon incubation with obidoxime is considered to
310 have arisen from a dealkylation reaction that ages the modified enzyme (Worek, Thiermann et al.
311 2004). Mouse brain acetylcholinesterase was used in order to validate the protocol and compare the
312 nature of organophosphate mode of action in the two organisms (Kardos and Sultatos 2000).

313 **2.11. Statistical analysis**

314 Data were analysed using GraphPad Prism 7 and are given as mean ± SEM. Statistical significance was
315 assessed using two-way ANOVA followed by post hoc analysis with Bonferroni corrections when
316 applicable. Bonferroni corrections were selected to avoid false positives. The sample size N of each
317 experiment is specified in the figure.

318 # 3. Results

319 **3.1. Quantifying anti-cholinesterase induced changes in cholinergic neuromuscular function with**
320 **whole organism behaviour**

321 We first investigated distinct behaviours that are underpinned by cholinergic neuromuscular junction
322 function in *C. elegans*. This identified that locomotion/paralysis, contraction mediated shrinkage of

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323 body length and the rate of pharyngeal pumping showed a clear concentration-time dependent
324 inhibition with respect to this class of anti-cholinesterase. The carbamate aldicarb was used as
325 representative of the acetylcholinesterase inhibitors. Similar to organophosphates, aldicarb binds and
326 inhibits acetylcholinesterase, resulting in the increase of acetylcholine concentration at the
327 neuromuscular junctions (Colovic, Krstic et al. 2013). This produces an overstimulation of the
328 cholinergic receptors that leads to the hypercontraction of the muscle cells at different neuromuscular
329 junctions in *C. elegans* (McVey, Mink et al. 2012). The aldicarb-induced hypercontraction of the body
330 wall muscles elicited both paralysis and decrease of the body length of the nematodes (Fig. 1).
331 However, the lowest concentrations of aldicarb tested (2 µM, 10 µM and 50 µM) failed to paralyse the
332 worms, even though the nematodes incubated on 50 µM aldicarb plates for 24 hours looked
333 uncoordinated and were significantly shorter than control nematodes (Fig. 1).

334 In a similar way, the aldicarb treatment caused a dose-dependent inhibition of pharyngeal pumping.
335 (Fig. 2). This paralysis is likely mediated by the elevated acetylcholine associated with cholinesterase
336 inhibition. The consequence is the hyperstimulation of the radial muscle contractions that underpin
337 rhythmic pumping mediated by a pacemaker cholinergic transmission (Avery 1993, Avery and Shtonda
338 2003, Niacaris and Avery 2003). This notion is supported by experiments in which the isolated pharynx
339 is exposed to physiological Dent's solution with or without 5 µM aldicarb for 5 min (Fig. 2B). In the
340 carbamate-treated isolated pharynx, we observed the predicted hypercontraction of the pharyngeal
341 radial muscle evidence by the sustained opening of the pharyngeal lumen (Fig. 2B). In contrast to body
342 wall muscles, the whole organism paralysis of the pharyngeal muscle was observed with the lowest
343 concentrations tested within 6 hours of incubation on the aldicarb-containing plate and from the first
344 hour of intoxication onto 50 µM plates.

345 The IC50 values calculated after 24 hours of intoxication indicated that pharyngeal pumping rate and
346 body length were the most sensitive behaviours to anti-cholinesterase intoxication, being 5 fold lower
347 than paralysis IC50 value (Fig. 3). Moreover, the pharyngeal pumping rate discriminated the
348 incremental effect of increasing concentrations of cholinesterase inhibition. Furthermore, this whole
349 organism bio-assay of cholinergic neuromuscular junction was more sensitive than body length in
350 resolving the low concentration as well as discerning anti-cholinergic effects at shorter incubation
351 times.

352 **3.2. *C. elegans* acetylcholinesterase activity is reduced by the presence of aldicarb.**

353 In order to demonstrate that aldicarb inhibits acetylcholinesterase activity in the treated *C. elegans*,
354 intoxicated worms on 50 µM and 500 µM aldicarb plates were harvested and homogenized. The
355 acetylcholinesterase activity in the homogenates was measured and compared with non-treated

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356 animals (Fig. 4A). Nematodes intoxicated onto aldicarb plates for 24 hours exhibited a reduction in the
357 acetylcholinesterase activity that was dependent on the inhibitor concentration (Fig. 4A). Interestingly,
358 the inhibition of enzyme activity was greater than the reduction in any of the neuromuscular junction
359 dependent phenotypes investigated. The nematodes incubated on 50 µM aldicarb displayed 15% of
360 acetylcholinesterase activity found in control homogenates (Fig. 4A). In contrast, locomotion, body
361 length and pharyngeal pumping were 100%, 83% and 53%, respectively, compared to the
362 corresponding control (Fig. 4B, 4C, 4D). Similarly, the acetylcholinesterase activity of nematodes after
363 24 hours of incubation on 500 µM aldicarb plates was 3.4% while the ratio of worms moving, body
364 length and pumping rate was 14.6%, 53.9% and 2.5%, respectively, compared to the corresponding
365 control of non-treated worms (Fig. 4). These data indicate the high safety factor associated with
366 cholinesterase function in which low levels of acetylcholinesterase activity can maintain the
367 behavioural function in addition with a large reserve of acetylcholinesterase available to replace the
368 organophosphate inhibited. This is consistent with previous data from mammal models in which the
369 function was maintained despite profound enzyme inhibition (Wolthuis, Groen et al. 1995).

370 **3.3. Pharyngeal microcircuits are more sensitive to irreversible acetylcholinesterase inhibitors than**
371 **to the carbamate aldicarb**

372 In order to test the effect of irreversible organophosphate anti-cholinesterase inhibitors in the
373 pharynx of *C. elegans*, concentration-time dependent curves were generated for pumping rate in the
374 presence of paraoxon-ethyl, paraoxon-methyl and diisopropylfluorophosphate (DFP) (Fig. 5).
375 Paraoxon-ethyl and –methyl were used as representative of organophosphate pesticides. In mammals,
376 both compounds exhibit similar acetylcholinesterase inhibition rate constants (Worek, Thiermann et
377 al. 2004). In contrast, the spontaneous hydrolysis and aging constants for them are distinct, indicating
378 paraoxon-methyl is more likely to age the enzyme after inducing inhibition (Worek, Thiermann et al.
379 2004). DFP was used as representative of organophosphate nerve agents. DFP exhibits a higher
380 inhibition and lower aging constant of mammal acetylcholinesterase compared to the paraoxon
381 derivatives. Unlike these compounds, DFP does not show spontaneous hydrolysis, facilitating its use
382 as a nerve agent (Worek, Thiermann et al. 2004).

383 The potency of all the organophosphates to inhibit pharyngeal pumping (Fig. 5) was much greater than
384 observed with the carbamate (Fig. 2 and 3). The potency of inhibition of pharyngeal pumping was
385 similar for each organophosphate. The estimated IC50 values for paraoxon-ethyl, DFP and paraoxon-
386 methyl were 15.52 µM, 16.18 µM and 24.04 µM, respectively (Fig. 5A, 5B and 5C).

387 **3.4. *C. elegans* acetylcholinesterase activity is reduced by the presence of organophosphate**
388 **compounds.**

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389 To characterise the inhibition of *C. elegans* acetylcholinesterase by the paraoxon derivatives and DFP,
390 organophosphates were added to untreated worm protein. To benchmark this approach, parallel
391 experiments using mouse brain homogenates were run. Acetylcholinesterase activity from the protein
392 homogenates was quantified after the exposure of a single concentration of the organophosphate at
393 increasing times of incubation (Fig. 6).

394 Paraoxon-ethyl inhibition over time showed two different phases in both the *C. elegans* and mouse
395 homogenate (Fig. 6A and 6B). The inhibition rate was greater at the first times of intoxication than in
396 later exposure times.

397 Upon incubation with DFP, both the nematode and mouse acetylcholinesterase activity was
398 diminished within the first 2 minutes of exposure, after which it levelled off to a steady state inhibition
399 (Fig. 6C and 6D). However, the steady state inhibition of worm acetylcholinesterase reached after 2
400 minutes of incubation with DFP was about 60% of the total activity while the inhibition of mouse
401 acetylcholinesterase was only 20%. It might indicate that *C. elegans* acetylcholinesterase is more
402 susceptible for DFP inhibition than the mouse enzyme in the conditions assayed.

403 Paraoxon-methyl inhibition over the time in *C. elegans* and mouse homogenate fitted a single decay
404 curve. Nevertheless, the inhibition of worm acetylcholinesterase is progressive over the time while
405 the reduction of the mouse acetylcholinesterase activity is more rapid, reaching steady state within 1
406 min of incubation (Fig. 6E and 6F).

407 Overall, the reduction of the pharyngeal pumping rate in the presence of the anti-cholinesterases was
408 associated with a time and concentration dependent inhibition of the nematode acetylcholinesterase.
409 Based on the degree of organophosphate-induced inhibition of behaviour and homogenate associated
410 acetylcholinesterase activity, the results suggest that DFP is more efficient than paraoxon-ethyl, which
411 is more efficient than paraoxon-methyl, even if the IC50 values and homogenate inhibition reached
412 similar values at the longest incubation time (Fig. 5 and 6).

413 **3.5. Recovery of pharyngeal function from organophosphates intoxication**

414 To investigate if pharyngeal pumping recovered from anti-cholinesterase intoxication, nematodes
415 were incubated with inhibitors for 24 hours. The intoxicated nematodes were then transferred onto
416 control plates, or ones treated with obidoxime or pralidoxime. These oximes, which did not affect
417 pumping themselves, were investigated to see if their known ability to facilitate recovery is manifest
418 in *C. elegans* (Fig. 7).

419 Nematodes intoxicated with 100 µM paraoxon-ethyl exhibited a fast recovery of the pharyngeal
420 function, which was complete at 4 hours after being transferred onto empty plates (Fig. 7B). Recovery

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421 was accelerated when nematodes were removed from organophosphate and placed onto either the
422 obidoxime or the pralidoxime plates. Obidoxime was the more effective of the two compounds tested
423 in rescuing the pharyngeal activity, with a half-time of recovery of 1 h compared to 1.33 h on
424 pralidoxime or 2 h on control plates (Fig. 7B). In contrast, nematodes incubated on either 100 µM
425 paraoxon-methyl or 250 µM DFP plates for 24 hours did not show complete recovery when transferred
426 to drug free plates (Fig. 7C and 7D). Moreover, the exposure of intoxicated worms to either of the
427 oximes tested did not improve the rescue of the pharyngeal function.

428 Overall, the complete, fast and oxime-sensitive recovery of the pharyngeal function after paraoxon-
429 ethyl exposure indicates that paraoxon-ethyl is not able to age the worm acetylcholinesterase and the
430 presence of oximes facilitates the reactivation of the inhibited enzyme. In contrast, the slow,
431 incomplete and oxime-insensitive recovery of the pumping rate after either paraoxon-methyl or DFP
432 intoxication suggests these compounds irreversibly inhibit *C. elegans* acetylcholinesterase.

433 **3.6. Nematode acetylcholinesterase recovery after organophosphate inhibition**

434 To test if the organophosphates that inhibit pharyngeal pumping irreversibly modify the worm
435 acetylcholinesterase, we investigated the recovery of the organophosphate-inhibited homogenates
436 with and without the post intoxication addition of obidoxime.

437 Both worm and mouse acetylcholinesterase inhibited by paraoxon-ethyl had a significant but
438 incomplete recovery by obidoxime (Fig. 8A and 8B).

439 As already observed (Fig. 6C and 6D), the inhibition of worm acetylcholinesterase by DFP was greater
440 than the inhibition of mouse acetylcholinesterase (Fig. 8C and 8D). After the incubation step with
441 obidoxime, there was no significant recovery of the acetylcholinesterase activity supporting enzyme
442 inhibition by this organophosphate had progressed through an irreversible reaction.

443 Finally, worm/mouse acetylcholinesterase exposed to paraoxon-methyl exhibited a nearly complete
444 reduction of the activity (Fig. 8E and 8F). The presence of obidoxime did not improve the
445 acetylcholinesterase activity of the mouse paraoxon-methyl enzyme indicating a rapid irreversible
446 inhibition (Fig. 8F). Nonetheless, the effect of obidoxime in the worm paraoxon-methyl inhibited
447 acetylcholinesterase indicated a time-dependent process. At 30 min after removing the excess of
448 organophosphate, the presence of obidoxime recovered the total acetylcholinesterase activity,
449 indicating a reversible reaction of inhibition at this time point. At subsequent times beyond 30 min,
450 there was no improvement in the acetylcholinesterase activity of paraoxon-methyl inhibited enzyme
451 by the incubation with obidoxime (Fig. 8E). This is consistent with a progressed inhibition through a
452 classic aging reaction (Sun, Chang et al. 1979) or alternatively an inability of oxime to execute a

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453 nucleophilic attack at the organophosphate bound to the serine at the active site (Worek, Thiermann
454 et al. 2004, Worek, Thiermann et al. 2016).

455 # 4. Discussion

456 **4.1. Pharyngeal pumping rate as mechanism for evaluating the effect of anti-cholinesterase**
457 **intoxication**

458 In the present study, we have used whole organism intoxication of *C. elegans* to investigate carbamate
459 and organophosphate poisoning of cholinesterase. The study verifies previous results that worm
460 behaviours are dependent on cholinergic transmission and therefore suitable to investigate anti-
461 cholinesterase intoxication (Cole, Anderson et al. 2004, Boyd, McBride et al. 2007, Rajini, Melstrom et
462 al. 2008, McVey, Mink et al. 2012, Leelaja and Rajini 2013). Most of the previous studies were focused
463 on the direct effect of anti-cholinesterases on *C. elegans* movement, either in liquid or solid culture,
464 while the pharyngeal effect has been indirectly scored by the reduction of food availability in liquid
465 culture (Boyd, McBride et al. 2007, Rajini, Melstrom et al. 2008, Boyd, Smith et al. 2010). However,
466 the recovery of those behaviours after acetylcholinesterase inhibitors exposure has never been
467 probed. This is an important area for investigating mitigation approaches and cross-referencing to the
468 similarity of core mode of action in the model organisms and humans.

469 The pharyngeal pump depends on acetylcholine excitation of the pharyngeal muscles to drive the
470 contraction and relaxation cycle that allows the food intake (Avery 1993, McKay, Raizen et al. 2004,
471 Boyd, McBride et al. 2007). This readily scored behaviour on plates offers a distinct route to test
472 acetylcholinesterase intoxication and recovery. Comparing the sensitivity of the pump rate to
473 intoxication relative to the shrinkage of the worm or the binary scoring of paralysis suggests this assay
474 may be more sensitive and better suited to discern the incremental concentration-dependent and
475 recovery effects. The assay, which is conducted on the worms on food, has a good dynamic range.
476 Pumping is elevated from about 40 to 250 pumps per minute when worms enter the food and the
477 concentration-dependent inhibition of this activity by the anti-cholinesterase appears to operate
478 across this dynamic range. Indeed, when judged as an observer based bio-assay, it is more sensitive
479 than locomotion, previously described as a phenotype for assessing organophosphate intoxication
480 (Cole, Anderson et al. 2004, Melstrom and Williams 2007, Leelaja and Rajini 2013). The pharyngeal
481 neuromuscular innervation of *C. elegans* consists of a subset of cholinergic and glutamatergic neurons
482 that synapse onto the radial muscles of the pharynx (Albertson and Thomson 1976, Trojanowski,
483 Raizen et al. 2016). The release of acetylcholine mainly by the MC and M4 motor neurons results in
484 the contraction of the muscles causing the opening of the lumen and therefore the entering of bacteria
485 (Albertson and Thomson 1976, Trojanowski, Raizen et al. 2016). In the presence of anti-cholinergic

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486 compounds, the pharyngeal muscles remain hypercontracted and the lumen continuously open (Fig.
487 2B) causing the paralysis of the pharyngeal movement (Fig. 2A). We demonstrated that the reduction
488 of the pumping rate was better correlated with the inhibition of the acetylcholinesterase activity by
489 aldicarb compared to body wall neuromuscular junction phenotypes (Fig. 4). This fact might be due to
490 a differential sensitivity of the pharyngeal circuits to intoxication with acetylcholinesterase inhibitors
491 compared to body wall circuits. The ingestion of the anti-cholinesterase compounds with the bacteria
492 while feeding might be a faster access pathway for the inhibitors than throughout the cuticle. However,
493 the pharyngeal movement quantification is also a better discriminatory assay, ranging the impact of
494 intoxication from 0 to 250 pumps/min while there is not such an incremental effect in the paralysis of
495 the locomotion.

496 The intoxication of the pharyngeal muscles by organophosphates caused a reduction of the pumping
497 rate, which gave the rank order of potency of toxicity: paraoxon-ethyl > DFP > paraoxon-methyl with
498 slight differences of the IC50 values between them (Fig. 5). Similar to mammalian investigations, the
499 acute toxicity of organophosphates was associated with the block of the acetylcholinesterase activity
500 by the inhibitors (Fig. 6). In fact, the biochemical reduction of acetylcholinesterase activity during
501 exposure indicates a similar ranking of toxicity as the one measured with pharyngeal pumping (Fig. 5,
502 6A, 6C, 6E). It might indicate that organophosphates can easily access the worm acetylcholinesterases
503 when the nematodes are on inhibitor-containing plates. They block enzyme activity, causing the
504 hypercontraction of the pharyngeal muscles and therefore the paralysis of the feeding. The route of
505 the drug access into the worm is still unknown; it might be through the cuticle but also by ingestion
506 when they feed.

507 The action of organophosphates in the mouse homogenate indicates that acetylcholinesterase is more
508 susceptible to the inhibition by either paraoxon-ethyl or -methyl than by DFP (Fig. 6B, 6D, 6F). This is
509 consistent with acute toxicity and kinetic data previously published for murine models poisoned with
510 organophosphates where LD50 values and inhibition constants for DFP are slightly higher than for
511 paraoxon-ethyl or –methyl, independently of the mode of administration (Johnson and Wallace 1987,
512 Gearhart, Jepson et al. 1990, Misik, Pavlikova et al. 2015).

513 The different rank of toxicity for DFP between *C. elegans* and mouse acetylcholinesterase might
514 indicate a difference in the kinetics of inhibition by the OP between the two organisms. This variance
515 has been previously described among the diverse organism models probed for their reactivity
516 acetylcholinesterase inhibition and recovery (Johnson and Wallace 1987, Gearhart, Jepson et al. 1990,
517 Worek, Thiermann et al. 2004, Worek, Aurbek et al. 2008, Coban, Carr et al. 2016). Despite the
518 different rank of toxicity, both *C. elegans* and mouse DFP-inhibited acetylcholinesterase exhibited no

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519 recovery after obidoxime treatment (Fig. 8C and 8D), which is consistent with the absence of recovery
520 observed in the pharyngeal pumping after DFP intoxication (Fig. 7C).

521 **4.2. Pharyngeal pumping rate as a metric for evaluating spontaneous recovery and reactivation after**
522 **organophosphate intoxication**

523 The recovery of the acetylcholinesterase activity is key to treat the cholinergic syndrome. Oxime
524 treatment in humans after OP poisoning offers an established supporting therapy (Eddleston and
525 Chowdhury 2016). However, the recovery and the oxime efficiency is an OP-dependent process
526 (Worek, Thiermann et al. 2004). In mammalian models, the rate of the reaction and the efficiency of
527 possible therapies can be analysed biochemically by quantifying the acetylcholinesterase activity
528 either in blood or brain samples of intoxicated animals (Maxwell, Brecht et al. 1987, Bajgar 1992, Misik,
529 Pavlikova et al. 2015). We describe here a simple *in vivo* experiment in a model organism that is
530 potentially indicative of the chemical state of the acetylcholinesterase active site after the
531 organophosphate intoxication. The recovery of the pharyngeal function after paraoxon-ethyl
532 exposure and the improvement by the oximes (Fig. 7B) supports an oxime-sensitive reaction between
533 the OP and the worm acetylcholinesterase (Fig. 8A). In biochemical experiments using worm protein,
534 we observed a reduction of acetylcholinesterase activity that can be rescued by the incubation with
535 obidoxime (Fig. 8A). In contrast, the inefficiency of the oxime treatment after paraoxon-methyl or DFP
536 inhibition indicates an irreversible modification of the nematode enzyme by these organophosphates
537 (Fig. 8C and 8E). This was manifest by the failure to recover pharyngeal function when intoxicated
538 worms are transferred onto either empty or oxime-containing plates (Fig. 7C and 7D).

539 The biochemical study of spontaneous recovery and oxime-sensitive reaction in mouse
540 acetylcholinesterase is consistent with previously published data (Tripathi and Dewey 1989, Kardos
541 and Sultatos 2000). Non-aged acetylcholinesterase after the exposure of paraoxon-ethyl was able to
542 recover partially the activity in the presence of obidoxime (Fig. 8B) while the aged acetylcholinesterase
543 that predominates after either paraoxon-methyl or DFP inhibition could not be rescued by the oxime
544 treatment (Fig. 8D and 8F) (Tripathi and Dewey 1989, Kardos and Sultatos 2000).

545 To conclude, the analysis of the nematode pharyngeal function after OP intoxication might be
546 indicative of the acetylcholinesterase state after the enzyme inhibition, spontaneous and obidoxime-
547 induced reactivation.

548 5. Conclusion

549 In previous studies, *C. elegans* body wall phenotypes have been used to understand
550 acetylcholinesterase inhibition by organophosphate exposure and, in some of them; it was correlated
551 with the quantification of acetylcholinesterase activity in the worm (Melstrom and Williams 2007,

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552 Rajini, Melstrom et al. 2008, Leelaja and Rajini 2013). Here, we demonstrated in the present study
553 that the pharyngeal function represents a more precise phenotype to understand acetylcholinesterase
554 inhibition by OP drugs. Interestingly, the rescue of the phenotype was also correlated with the rate of
555 the acetylcholinesterase reaction upon OP inhibition as well as the efficiency of the reactivators. It
556 makes the pharynx of *C. elegans* an attractive tool for discovering new drugs able to reactivate the
557 inhibited acetylcholinesterase. Furthermore, clear benchmarking of this class of neurotoxicological
558 agents in a tractable bio-assay in *C. elegans* means that genetic manipulation of these effects can be
559 probed. This provides a new approach to investigate mitigation of such neurotoxicity that may
560 translate to human poisoning.

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568 **Author contributions**

569 **Patricia G. Izquierdo**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology,
570 Validation, Visualization, Roles/Writing - original draft. **Vincent O'Connor**: Conceptualization, Funding
571 acquisition, Methodology, Supervision, Writing - review & editing. **Christopher Green**:
572 Conceptualization, Funding acquisition, Methodology, Supervision, Writing - review & editing. **Lindy**
573 **Holden-Dye**: Conceptualization, Funding acquisition, Methodology, Supervision, Writing - review &
574 editing. **John Tattersall**: Conceptualization, Funding acquisition, Methodology, Supervision, Writing -
575 review & editing.

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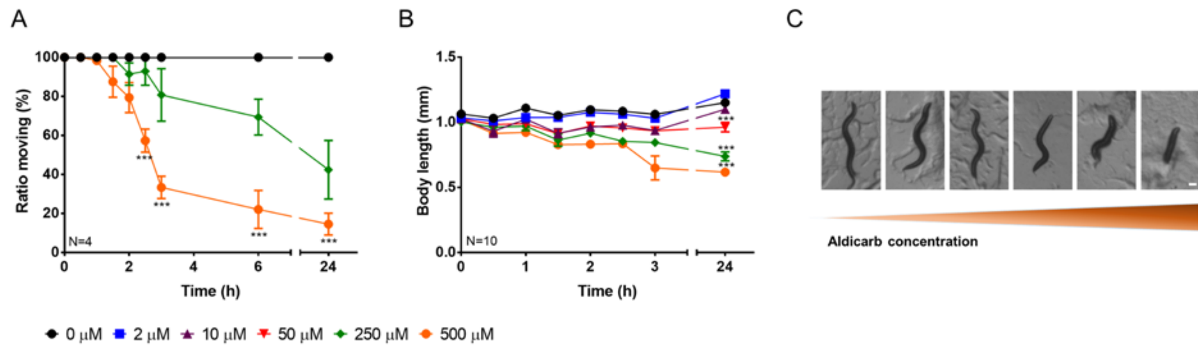

576

577 **Figure 1. Nematodes exposed to aldicarb exhibited paralysis and hypercontraction of body wall**
578 **muscles.** A) The number of synchronized L4 + 1 nematodes moving as percentage the total worms on
579 the plate was scored at different times in the face of a range of concentrations of aldicarb. The ratio
580 moving of nematodes exposed to 2 µM, 10 µM and 50 µM was identical to the control. Data are shown
581 as mean ± SEM of four different experiments. B) The body length of synchronized L4 + 1 nematodes
582 exposed to different concentrations of aldicarb plates was scored by taking micrographs at different
583 times of incubation and the length quantified. Data are shown as mean ± SEM of the length of ten
584 worms in five different experiments. C) Body length of nematodes incubated on different
585 concentrations of aldicarb plates for 24 hours. From the left to the right the concentration of aldicarb
586 was: 0 µM (control), 2 µM, 10 µM, 50 µM, 250 µM and 500 µM. Scale bar represents 100 µm. *p<0.05;
587 **p<0.01; ***p<0.001 by two-way ANOVA test.

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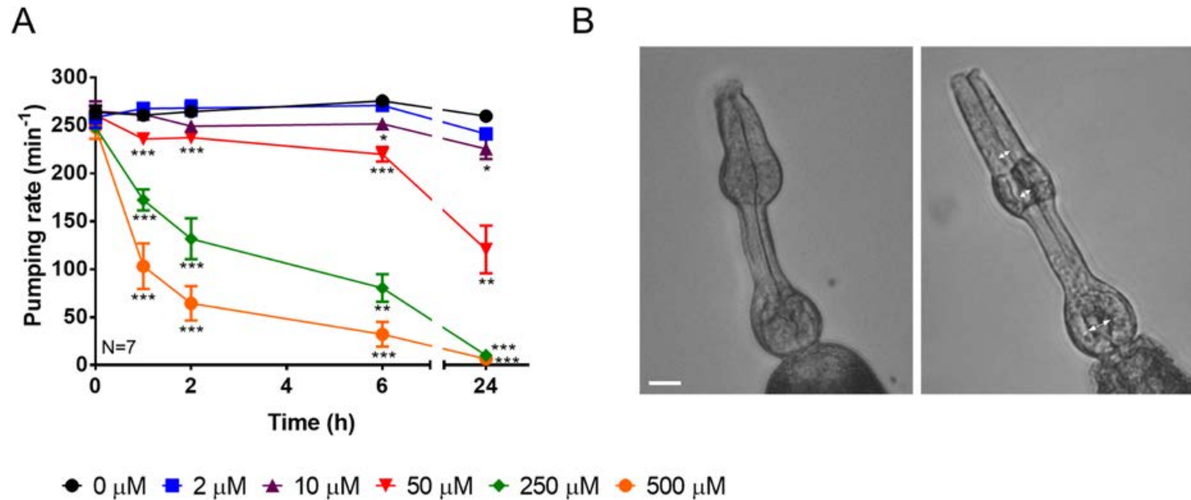

588

589 **Figure 2. Pharyngeal pumping of *C. elegans* exposed to aldicarb exhibited a gradual concentration-**
590 **time dependent paralysis due to the hypercontraction of the radial muscles in the pharynx.** A)
591 Pharyngeal pumping rate per minute was quantified at different end-point times for synchronized L4
592 + 1 nematodes exposed to a range of concentration of aldicarb plates. An increased concentration-
593 dependent response over the time is observed. Data are shown as mean ± SEM of the pumping rate
594 of 7 worms in four different experiments. B) Isolated pharynx of *C. elegans* were exposed to Dent's
595 solution as control (left panel) or 5 µM of aldicarb (right panel). The hypercontraction of the radial
596 muscles caused the opening of the pharyngeal lumen (indicated by the white arrows). Scale bar
597 represents 1 µm. *p<0.05; **p<0.01; ***p<0.001 by two-way ANOVA test.

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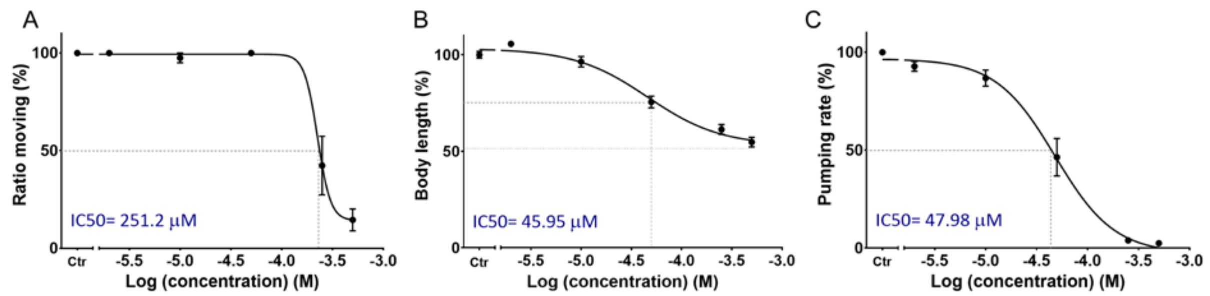

599

600 **Figure 3. Aldicarb concentration-dependent sensitivity in cholinergic neuromuscular junction**
601 **dependent behaviours.** A) The percentage of ratio moving corresponds to the number of worms
602 moving out of the total number of worms on the plates after 24 hours of intoxication. B) Body length
603 was expressed as percentage of the unexposed body length after 24 hours of incubation. C) Pharyngeal
604 pump rate was expressed as percentage of the pharyngeal pumping of unexposed nematodes after
605 24 hours of incubation. Data are shown as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001 by two-way
606 ANOVA test.

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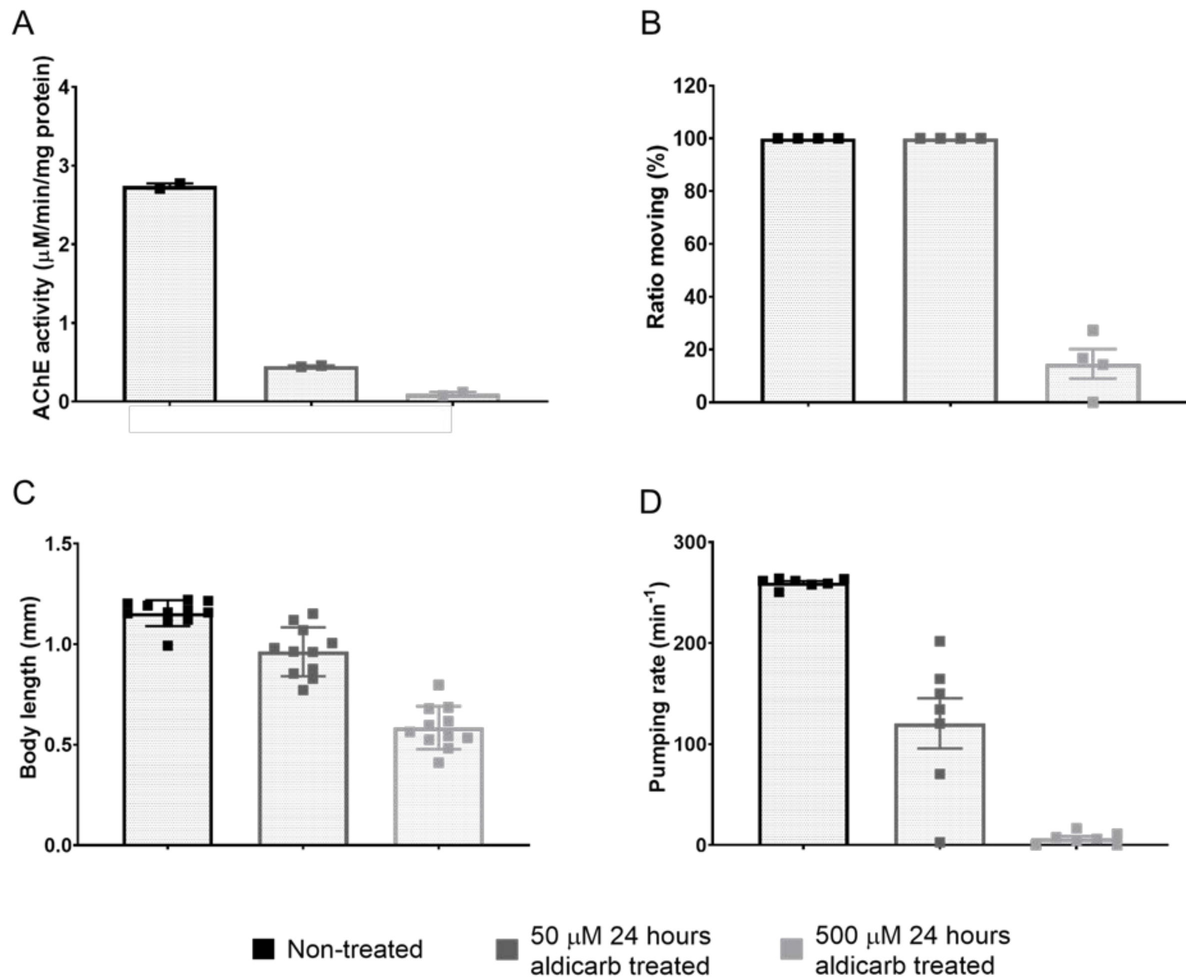

607

608 **Figure 4. *C. elegans* acetylcholinesterase activity associated with reduced pharyngeal pumping rate**
609 **and motility behaviours after 24 hours of intoxication.** A) *C. elegans* acetylcholinesterase activity
610 associated with homogenates from synchronized L4/adult worms isolated after 24 hours of incubation
611 onto empty, 50 µM and 500 µM aldicarb plates. Treated worms were homogenized and enzyme
612 activity measured using a modified Ellman's assay. Data are shown as mean ± SEM of two independent
613 experiments. B) The ratio moving was scored as the animals moving after 24 hours of exposure onto
614 empty, 50 µM and 500 µM aldicarb plates as percentage of the total worms on the corresponding
615 plate. Data are shown as mean ± SEM of four independent experiments. C) Body length of L4 + 1
616 nematodes was scored after 24 hours exposed to 50 µM, 500 µM aldicarb or unexposed. Data are
617 shown as mean ± SEM of the length of 10 worms in five independent experiments. D) Pharyngeal
618 pumping rate of unexposed, 50 µM and 500 µM aldicarb exposed L4+1 synchronized nematodes after
619 24 hours of intoxication. Data are shown as mean ± SEM of seven worms in four independent
620 experiments.

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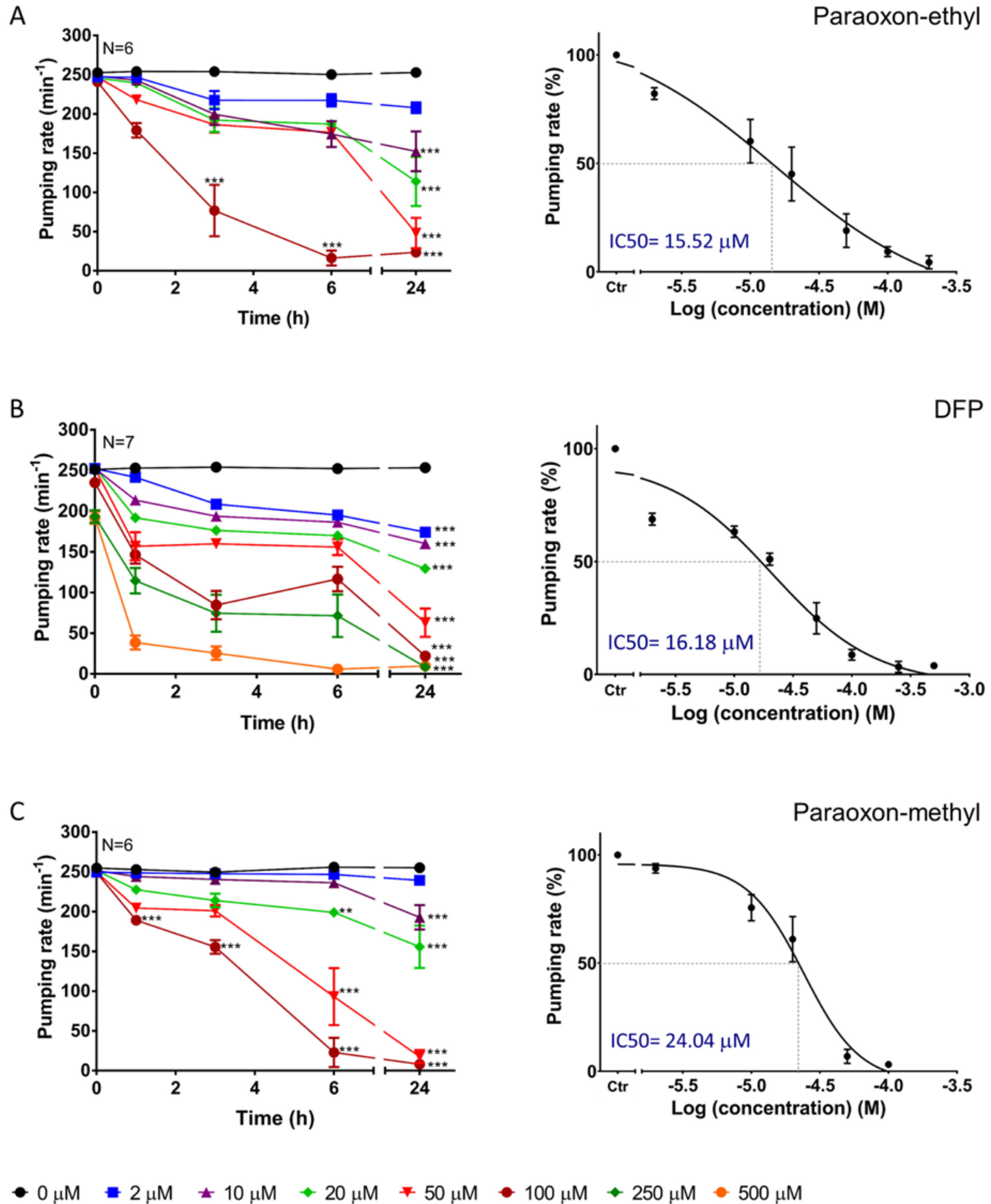

621

622 **Figure 5. Pharyngeal pumping of *C. elegans* exposed to organophosphates.** Pharyngeal pumping rate
623 was quantified at indicated times with a range of concentrations of paraoxon-ethyl (A), DFP (B) and
624 paraoxon-methyl (C). The IC50 values were calculated from the pump rate recorded at 24 hours of
625 exposure to drug relative to untreated vehicle control. Data are shown as mean ± SEM of 6/7 worms
626 in four independent experiments. *p<0.05; **p<0.01; ***p<0.001 by two-way ANOVA test.

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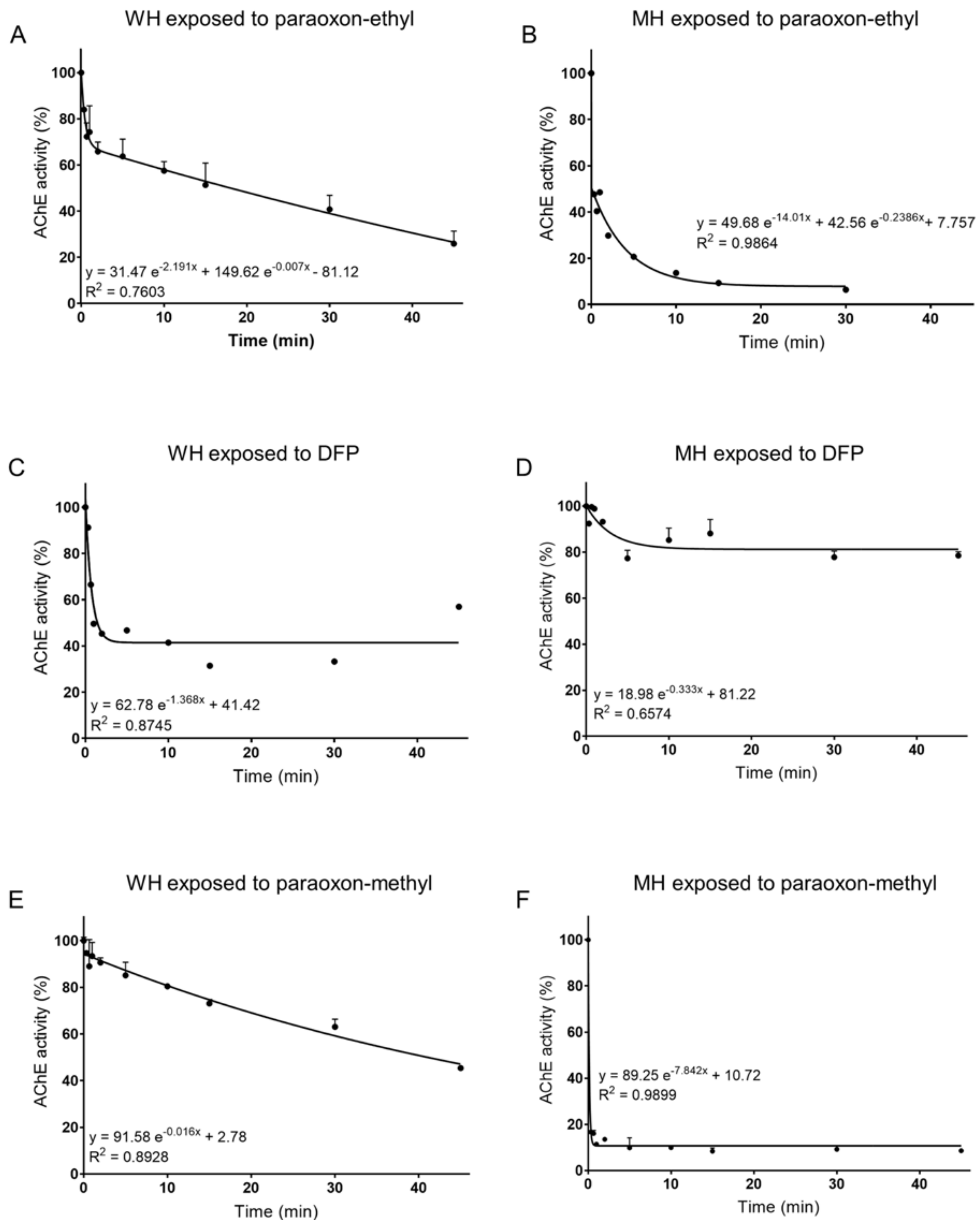

627

628 **Figure 6. Paraoxon-ethyl, DFP or paraoxon-methyl show a time dependent inhibition of the**
629 **acetylcholinesterase activity associated with *C. elegans* and mouse brain homogenates.** Worm (WH)
630 and mouse brain (MH) homogenates were incubated in addition of 1 µM of paraoxon-ethyl, DFP or
631 paraoxon-methyl to allow timed incubation of the enzyme inactivation before synchronized
632 measurement of homogenate associated acetylcholinesterase activity. Acetylcholinesterase activity
633 was expressed as percentage of the unexposed homogenate activity. Two-phase exponential decay
634 curve was ascribed as the best fit for the inhibition of nematode (A) and mouse (B)

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635 acetylcholinesterase activity at different end-point times of incubation with paraoxon-ethyl. Single
636 exponential decay curve was fitted to the inhibition of worm (C) and mouse (D) acetylcholinesterase
637 with DFP. One-phase inhibition of worm (E) and mouse (F) acetylcholinesterase inhibition with
638 paraoxon-methyl at different end-point times of incubation with the organophosphate.

639

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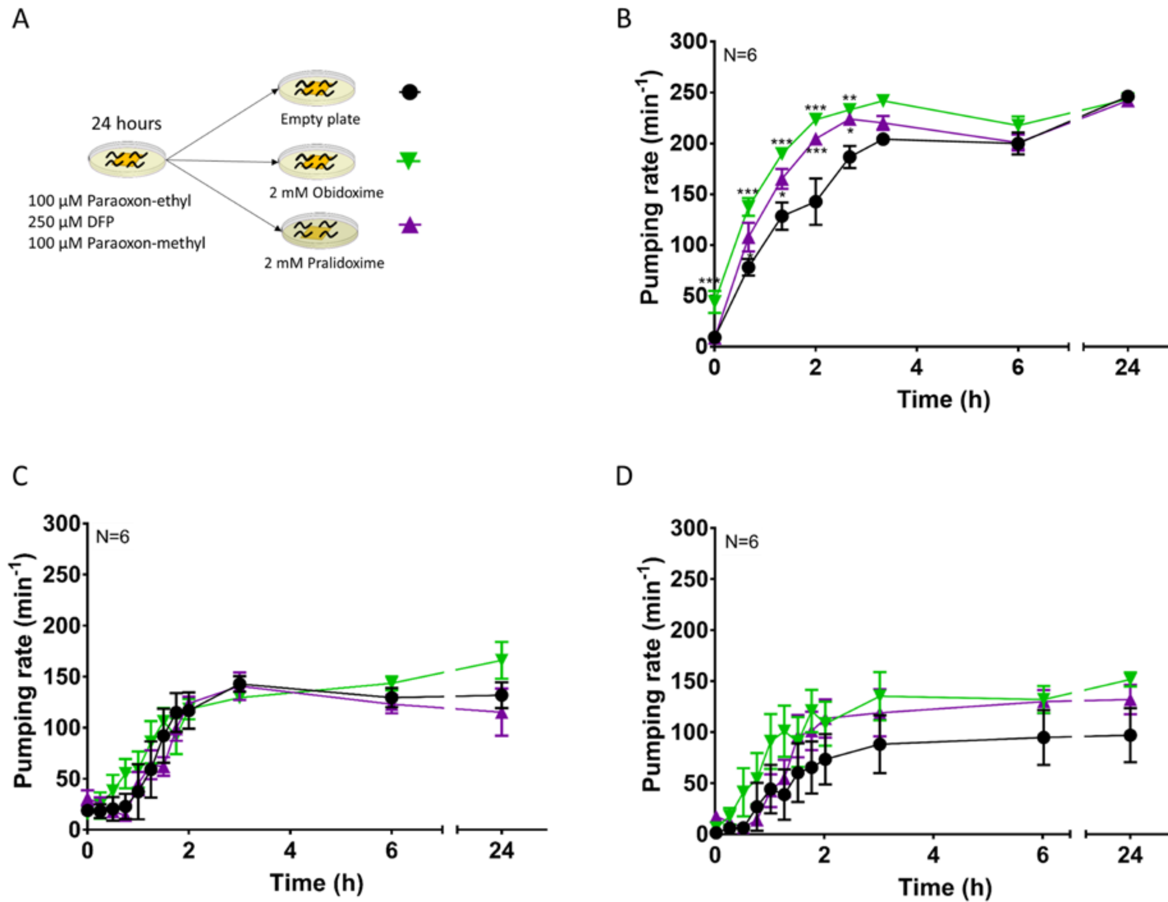

640

641 **Figure 7. Spontaneous and oxime induced recovery of pharyngeal pumping inhibition from**
642 **organophosphates intoxication.** A) Experimental design of pharyngeal function recovery after 100 µM
643 paraoxon-ethyl, 250 µM DFP or 100 µM paraoxon-methyl intoxication. Synchronized L4+1 nematodes
644 were incubated on drug-containing plates for 24 hours. After transfer to control, obidoxime or
645 pralidoxime containing plates pumping was scored at indicated times. B) Nematodes intoxicated on
646 paraoxon-ethyl plates exhibited a fast and complete recovery of pumping enhanced by oxime
647 treatment. C) Nematodes intoxicated on DFP plates for 24 hours displayed slow, incomplete and
648 oxime-insensitive recovery. D) Recovery from paraoxon-methyl was incomplete and oxime-
649 independent. Data are shown as mean ± SEM of six worms in six independent experiments. *$p<0.05$;
650 **$p<0.01$; ***$p<0.001$ by two-way ANOVA test.

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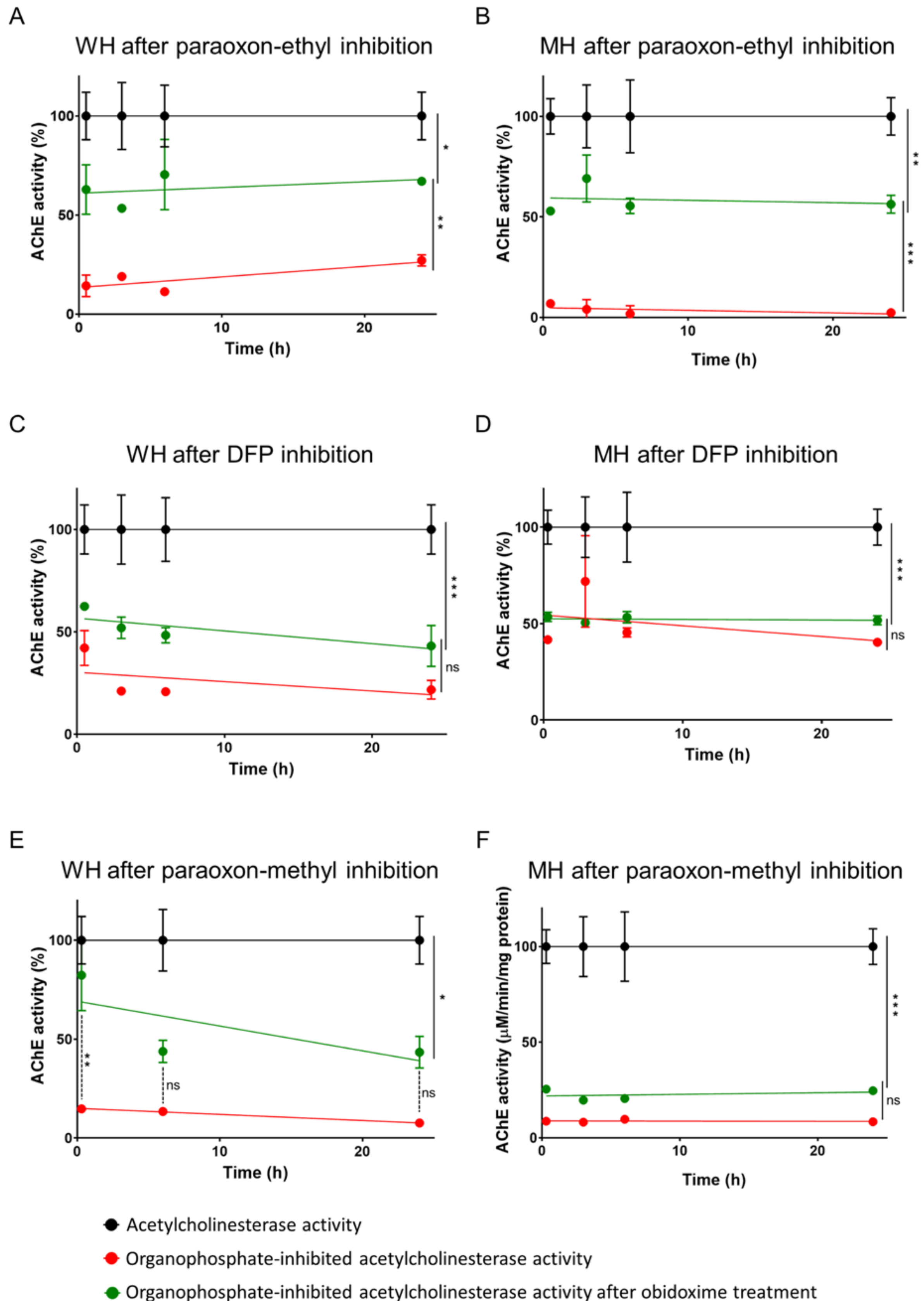

651

652 **Figure 8. *C. elegans* and mouse acetylcholinesterase is aged after the inhibition with either DFP or**

653 **paraoxon-methyl.** OP-inhibited worm (WH) or mouse (MH) acetylcholinesterase activity was

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654 quantified in the presence or absence of a single concentration of obidoxime. Untreated homogenates
655 were used as controls. Acetylcholinesterase activities were represented as the percentage of activity
656 referred to the untreated control. A) Worm homogenate inhibited by paraoxon-ethyl exhibited an
657 80% reduction of the acetylcholinesterase activity that was partially recovered by the obidoxime
658 treatment. B) Mouse homogenate acetylcholinesterase inhibited by paraoxon-ethyl displayed a partial
659 recovery of its activity after obidoxime treatment. C) The obidoxime treatment did not significantly
660 improved the acetylcholinesterase activity of the worm acetylcholinesterase inhibited by DFP. D)
661 Mouse acetylcholinesterase activity was reduced after DFP treatment in 50%. Nevertheless, there is
662 not recovery of the enzyme activity after the obidoxime treatment. E) Worm homogenate exposed to
663 paraoxon-methyl displayed a reduction of the acetylcholinesterase activity that can be recovered by
664 the obidoxime treatment after 30 min of incubation. However, there is not recovery in the consequent
665 end-point times tested. F) The inhibition of mouse acetylcholinesterase activity by paraoxon-methyl
666 was not recovered by the obidoxime treatment. *$p<0.05$; **$p<0.01$; ***$p<0.001$ by two-way ANOVA
667 test.

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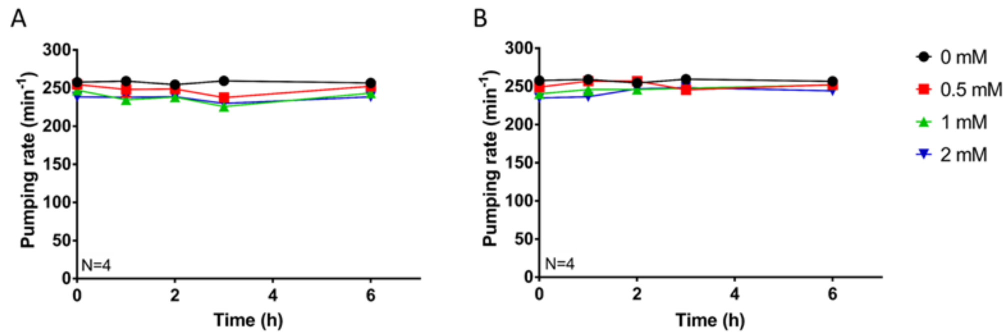

668

669 **Supplementary figure 1. Pharyngeal pumping rate phenotype of *C. elegans* wild type adults exposed**
670 **to oximes plates.** Pharyngeal pumping rate per minute was quantified at different end-point times for
671 synchronized L4+1 nematodes exposed to increasing concentration of plates containing obidoxime (A)
672 or pralidoxime (B). Neither obidoxime nor pralidoxime had any effect in the pharyngeal function of *C.*
673 *elegans*. Data are shown as mean ± SEM of four worms in four independent experiments.

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